# TISSUE SPECIFIC REGULATION OF 11ß-HYDROXYSTEROID DEHYDROGENASE BY ADRENAL STEROIDS, SEX STEROIDS AND GROWTH HORMONE.

**Susan C Low BSc.**

**A thesis submitted for the degree of Doctor of Philosophy**

**UNIVERSITY OF EDINBURGH**

**January 1994**

## DECLARATION

I declare that this thesis and the work presented here are entirely the result of my own independent investigation, apart from the procedures listed below which are also acknowledged in the text.
This work has not been and is not concurrently submitted for any other degree.

1. All surgical procedures and enzyme assays were carried out either by myself or with the help of June Noble of the Biomedical Research Facility, Western General Hospital.

2. Adjuvant injection in the adjuvant-induced arthritic model of stress, was carried out by Lucy Donaldson at the Department of Medicine, Western General Hospital.

3. Corticosterone binding globulin assays were performed by Isobel Forbes at the Department of Medicine, Western General Hospital.

4. Insertion of intravenous cannulae and pulsatile growth hormone infusions were carried out by Tim Wells at the NIMR, Mill Hill, London.

**ACKNOWLEDGEMENTS**

I would like to thank my supervisor, Jonathan Seckl who has always been enthusiastic and supportive about my work and has played a major role in my academic development over the past three years. I am also indebted to my "adopted" supervisor, Karen Chapman for two years of continual encouragement, guidance and discussion. A big thank you also to Paul Kelly who 'advised' me to do this PhD in the first place and was always there when I needed a shoulder.

Thank you to the Molecular Endocrinology group who are a good team to work with. I would especially like to thank Karen French who keeps the lab running smoothly and expertly teaches in situ hybridisation to all and sundry including me. A big thank you also goes to Keith and Andy for expert help with the animals.

I wish to thank and acknowledge the expert assistance of June Noble with animal surgery and maintenance, but also for coming to my rescue when enzyme assays were too much for me to cope with alone. June has also been extremely supportive and encouraging over the past three years and I am grateful to her for putting up with my troubles and woes.

Last but definitely not least, I would like to say thank you to my family - Thanks guys!

**Contents**

**Abstract**

Adrenal corticosteroid effects are mediated predominantly through intracellular receptors of two types, mineralocorticoid (MR) and glucocorticoid (GR). *In vitro,* MR bind aldosterone and the physiological glucocorticoids cortisol and corticosterone with equivalent affinities. However in kidney distal convoluted tubules and cortical collecting ducts, MR selectively bind aldosterone although circulating levels of glucocorticoids are 100 to 1000-fold in excess of aldosterone. This selectivity is due to the intracellular enzyme 11ß-hydroxysteroid dehydrogenase (11ß-OHSD) which catalyses the reversible conversion of physiological glucocorticoids (but not mineralocorticoids) to inactive products (cortisone and 11-dehydrocorticosterone), thus regulating their access to MR and perhaps GR. This thesis describes the tissue-specific regulation of 11ß-OHSD using an enzyme assay for total 11ß-OHSD activity, and northern analysis using a cDNA cloned from a rat liver library.

I have found that 11ß-OHSD activity and mRNA expression are regulated by glucocorticoids, but not mineralocorticoids, in rat hippocampus and liver, but not kidney. Hippocampal 11ß-OHSD activity and mRNA expression are elevated by dexamethasone and stress suggesting a role for 11ß-OHSD in modulating glucocorticoid access to neuronal MR. In agreement with this hypothesis, development of a double in-situ hybridisation technique demonstrated cellular co-localisation of 11ß-OHSD mRNA with MR and GR mRNAs in rat hippocampus.

I have also demonstrated that 11ß-OHSD activity exhibits a sexually-dimorphic pattern of expression in rat liver due to oestrogen repression in female rats which could not be explained by a direct action of oestrogen. However, hepatic 11ß-OHSD in dwarf rats exhibits attenuated sexual dimorphism suggesting that oestradiol effects may be mediated through secretory patterns of growth hormone (GH) which differ in male and female rats. Furthermore, continuous (female-type) administration of GH led to a decrease in hepatic 11ß-OHSD activity and mRNA expression which was not observed following pulsatile (male-type) GH treatment. Hippocampal 11ß-OHSD activity and mRNA expression were not affected by oestradiol or GH treatments supporting an indirect mechanism of oestradiol action, since sex steroids have free access to the brain, but GH, like many peptides, is unable to cross the blood-brain barrier. Hypophysectomy followed by oestradiol treatment revealed an additional direct action of oestradiol on hepatic 11ß-OHSD activity and mRNA expression which was not additive to the repression of hepatic 11ß-OHSD by GH. Hippocampal 11ß-OHSD was not affected by oestradiol treatment following hypophysectomy indicating that there are, as yet undetermined differences between hepatic and hippocampal 11ß-OHSD. In rat

kidney, 11ß-OHSD activity and mRNA expression were inversely regulated by sex steroids. Oestradiol treatment of male and female rats led to a marked increase in 11ß-OHSD activity in concurrence with almost complete suppression of 11ß-OHSD mRNA expression, providing evidence for the existence of a second dehydrogenase in rat kidney which is regulated in a complex manner by oestradiol and continuous but not pulsatile GH.

In order to understand the cellular role of the 'liver-type' 11ß-OHSD, a plasmid containing the coding region of the rat liver 11ß-OHSD cDNA linked to an SV40 promoter was transfected into COS-7 and CV-1 cells, and the conversion of [$^3$H] corticosterone to [$^3$H] 11-dehydrocorticosterone or [$^3$H] 11-dehydrocorticosterone to [$^3$H] corticosterone, monitored in the medium over a 24 hour period. Interestingly, it was found that in intact cells, the 'liver-type' 11ß-OHSD did not act as a dehydrogenase, but instead as a reductase (activating glucocorticoids), and therefore is highly unlikely to be the enzyme responsible for protecting MR in kidney tubules from exposure to glucocorticoids. This was confirmed at a molecular level using cells transfected with 11ß-OHSD, GR and MMTV-LTR luciferase reporter plasmids, which showed activation of otherwise inert 11-dehydrocorticosterone to induce the GR-dependent reporter gene. In conclusion, this thesis demonstrates tissue-specific regulation of 11ß-OHSD by adrenal steroids, sex steroids and growth hormone, provides evidence for the existence of a second dehydrogenase in rat kidney, and indicates that the 'liver-type' 11ß-OHSD is unlikely to be responsible for protecting renal MR from exposure to glucocorticoids.

**List of Figures**

**List of Tables**

**Commonly Used Abbreviations**

| | |
|---|---|
| 11ß-OHSD | 11 beta-hydroxysteroid dehydrogenase |
| A | 11-dehydrocorticosterone |
| ACTH | adrenal corticotrophic hormone |
| Adx | adrenalectomy |
| AEC | 3-amino-9-ethylcarbazole |
| AME | apparent mineralocorticodi excess |
| B | corticosterone |
| bp | base pair |
| CBG | corticosterone/cortisol binding globulin |
| cDNA | complimentary deoxyribonucleic acid |
| CRH | corticotrophin releasing hormone |
| cRNA | complimentary ribonucleic acid |
| DAB | 3,3'-diaminobenzidine |
| DEPC | diethyl pyrocarbonate |
| $dH_2O$ | distilled water |
| DMEM | Dulbecco's modification of Eagle's medium |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| DTT | dithiothreitol |
| Gdx | gonadectomy |
| GE | glycyrrhetinic acid |
| GI | glycglycyrrhizic acid |
| GH | growth hormone |
| GR | glucocorticoid receptor |
| GRH | growth hormone releasing factor |
| Hpx | hypophysectomy |
| MMTV-LTR | mouse mammary rumor virus long terminal repeat |
| MR | mineralocorticoid receptor |
| mRNA | messenger ribonucleic acid |
| NAD | nicotinamide adenine dinucleotide |
| NADP | nicotinamide adenine dinucleotide phoshphate |
| NADPH | nicotinamide adenine dinucleotide phosphate - reduced form |
| nt | nucleotide |
| ONPG | o-nitrophenyl-ß-D-galactopyranosidase |
| Ovx | ovariectomy |

| | |
|---|---|
| SS | somatostatin |
| SV40 | simian virus 40 |
| THE | tetrahydorcortisone |
| THF | tetrahydrocortisol |
| TLC | thin layer chromatography |
| X-Gal | 5-bromo-4-chloro-indolyl-b-D-galactosidase |

**Publications From This Thesis**

Full Papers

1. Benediktsson R, Yau JLW, Low SC, Brett L, Edwards CRW, Seckl JR. (1992) Ovarian 11ß-hydroxysteroid dehydrogenase: localisation to the oocyte. *J Endocrinol* **135**: 53-58.

2. Seckl JR, Dow RC, Low SC, Edwards CRW, Fink G. (1993) The 11ß-hydroxysteroid dehydrogenase inhibitor glycyrrhetinic acid affects corticosteroid feedback regulation of hypothalamic corticotrophin-releasing peptides. *J Endocrinol* **136**: 471-477.

3. Low SC, Assaad SN, Rajan V, Chapman KE, Edwards CRW, Seckl JR. (1993) Regulation of 11ß-hydroxysteroid dehydrogenase by sex steroids: Further evidence for a second dehydrogenase in rat kidney. *J Endocrinol* **139**: 27-35.

4. Low SC, Moisan M-P, Noble J, Edwards CRW, Seckl JR. Glucocorticoids regulate hippocampal 11ß-hydroxysteroid dehydrogenase activity and gene expression in vivo. *J Neuroendocrinol* (submitted).

5. Low SC, Chapman KE, Edwards CRW, Wells T, Robinson ICAF, Seckl JR. Sexual dimorphism of hepatic 11ß-hydroxysteroid dehydrogenase: The role of growth hormone patterns in the rat. *Endocrinol* (submitted).

6. Low SC, Chapman KE, Rossier BC, Edwards CRW, Seckl JR. Liver-type 11ß-hydroxysteroid dehydrogenase cDNA encodes reductase but not dehydrogenase activity in intact mammalian cells. *J Mol Endocrinol* (submitted).

Abstracts

1. Yau JLW, Low SC, Moisan M-P, Edwards CRW, Seckl JR. (1991) Localisation of 11ß-hydroxysteroid dehydrogenase mRNA expression in rat hypothalamus. *J Endocrinol* **129** (suppl): 234.

2. Seckl JR, Dow RC, Low SC, Edwards CRW, Fink G. (1991) 11ß-hydroxysteroid dehydrogenase mRNA expression in paraventricular nucleus: Enzyme inhibition reduces portal blood CRF. Society for Neuroscience: 434.11.

3. Low SC, Edwards CRW, Seckl JR. (1992) Effect of glucocorticoids on 11ß-hydroxysteroid dehydrogenase mRNA expression in rat hippocampus. *Scottish Med J* **37**: 91.

4. Low SC, Assaad SN, Edwards CRW, Seckl JR. (1992) Effect of sex steroids on 11ß-hydroxysteroid dehydrogenase in rat liver, kidney and hippocampus in vivo. *J Physiol* **452**: 76P.

5. Low SC, Moisan M-P, Assaad SN, Edwards CRW, Seckl JR. (1992) Steroid regulation of 11ß-hydroxysteroid dehydrogenase gene expression in hippocampus in vivo. *J Endocrinol* **132** (suppl): 52.

6. Moisan M-P, Low SC, Chapman KE, Edwards CRW, Seckl JR. (1992) Multiple tissue-specific isoforms of rat 11ß-hydroxysteroid dehydrogenase mRNA are due, in part, to differential promoter usage. 9th International Congress of Endocrinology: P535.

7. Seckl JR, Brown RW, Rajan V, Low SC, Edwards CRW. (1993) 11ß-hydroxysteroid dehydrogenase and corticosteroid actions in the brain. *J Endocrinol* **137** (suppl): S9.

8. Low SC, Chapman KE, Edwards CRW, Wells T, Robinson ICAF, Seckl JR. (1993) Do patterns of growth hormone secretion mediate the sexual dimorphism of hepatic 11ß-hydroxysteroid dehydrogenase? J Endocrinol 137 (suppl): OC30.

9. Low SC, Chapman KC, Edwards CRW, Wells T, Seckl JR. (1993) Sexual dimorphism of hepatic 11 beta-hydroxysteroid dehydrogenase is mediated indirectly via growth hormone. 75th Annual Meeting of the Endocrine Societies: 1398.

10. Chapman KE, Brown RW, Low SC, Edwards CRW, Seckl JR. (1993) Tissue-specificity of 11ß-hydroxysteroid dehydrogenase. J *Endocrinol* **139** (suppl): O7.

11. Low SC, Chapman KE, Edwards CRW, Seckl JR. (1994) Liver derived 11ß-hydrocysteroid dehydrogenase cDNA encodes reductase but not dehydrogenase activity in intact mammalian cells. *J Endocrinol* (submitted).

# CHAPTER 1

# INTRODUCTION

---

## 1.1 Adrenocortical Steroids

Steroid hormones are all derived from cholesterol, are synthesised in three tissues - the adrenal glands, the testis and the ovaries, and form four major classes - androgens, oestrogens, progestins and corticosteroids. Corticosteroids consist of mineralocorticoids and glucocorticoids, both of which are synthesised in the adrenal cortex and are metabolised in many other tissues. Mineralocorticoid and glucocorticoid levels are regulated at various levels including synthesis, metabolism, and exerting effects through two types of receptor - mineralocorticoid and glucocorticoid receptors. 11ß-hydroxysteroid dehydrogenase (11ß-OHSD) is also a tissue-specific regulator of corticosteroid levels, and is the subject of this thesis.

### 1.1.1 The Adrenal Cortex

Biosynthesis of glucocorticoids, mineralocorticoids and adrenal androgens occurs in the adrenal cortex which has been subdivided into three morphologically distinct zones, based on differences in vascular and connective tissues (Arnold et al., 1866). The outermost region, called the zona glomerulosa, lies just underneath the adrenal capsule and consists of clusters of compact cells which are involved in the synthesis of the major mineralocorticoid aldosterone. Cells in the middle layer, the zona fasiculata, are larger due to many lipid inclusions. Cells in the zona fasiculata may have extensions penetrating into the zona glomerulosa, and thus these two regions are not clearly demarcated. The innermost layer is the zona reticularis, which is clearly separated from the other two zones, and is comprised of loosely packed small cells which contain few lipid inclusions. The two innermost layers, although morphologically distinct, are both involved in the synthesis of the glucocorticoids cortisol and/or corticosterone, as well as producing relatively large amounts of the adrenal androgen dehydroepiandrosterone (DHEA) and its sulphate DHEAS.

### 1.1.2 Steroid Hormone Biosynthesis

The steroid hormones produced by the adrenal cortex are all derived from cholesterol, the basic structure of which is maintained throughout the steroid biosynthetic pathways (Fig. 1.1). Some of the steps involved in the pathways leading to synthesis of mineralocorticoids, glucocorticoids and adrenal androgens are shown in Fig. 1.2.

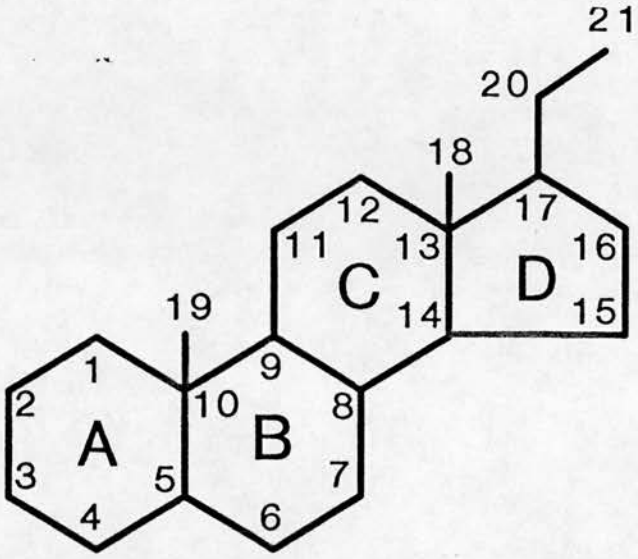



**Figure 1.1:**
Basic steroid ring structure. Numbers refer to the conventional designation of carbon atoms.

Four cytochrome P-450 enzymes, and an additional microsomal enzyme are involved in corticosteroid biosynthesis from cholesterol. Each enzymatic step is compartmentalised within the cell depending on the subcellular localisation of the enzyme involved. Firstly, cholesterol must be transported from the cytosol to the inner mitochondrial membrane, the site of cytochrome P-450$_{scc}$ (side chain cleavage) which cleaves the side chain of cholesterol at position C21 to form pregnenolone. This is the rate limiting step in steroidogenesis. Microsomal 3ß-hydroxysteroid dehydrogenase (which is probably not a cytochrome P-450 enzyme), is responsible for the conversion of the newly synthesised pregnenolone to progesterone. Pregnenolone and progesterone are substrates for two further enzymes associated with the smooth endoplasmic reticulum; P450$_{c17}$ (17α-hydroxylase) and P450$_{c21}$ (21-hydroxylase) which catalyse hydroxylations at positions C17 and C21 respectively. Mitochondrial P-450$_{c11}$ is responsible for hydroxylation of deoxycorticosterone and deoxycortisol to produce corticosterone and cortisol respectively. Thus the end products of corticosteroid biosynthesis fall into two main categories, the C21 steroids which have a two carbon side chain located at position C17, and the C19 steroids which contain either a hydroxyl group or a keto group at position C17.

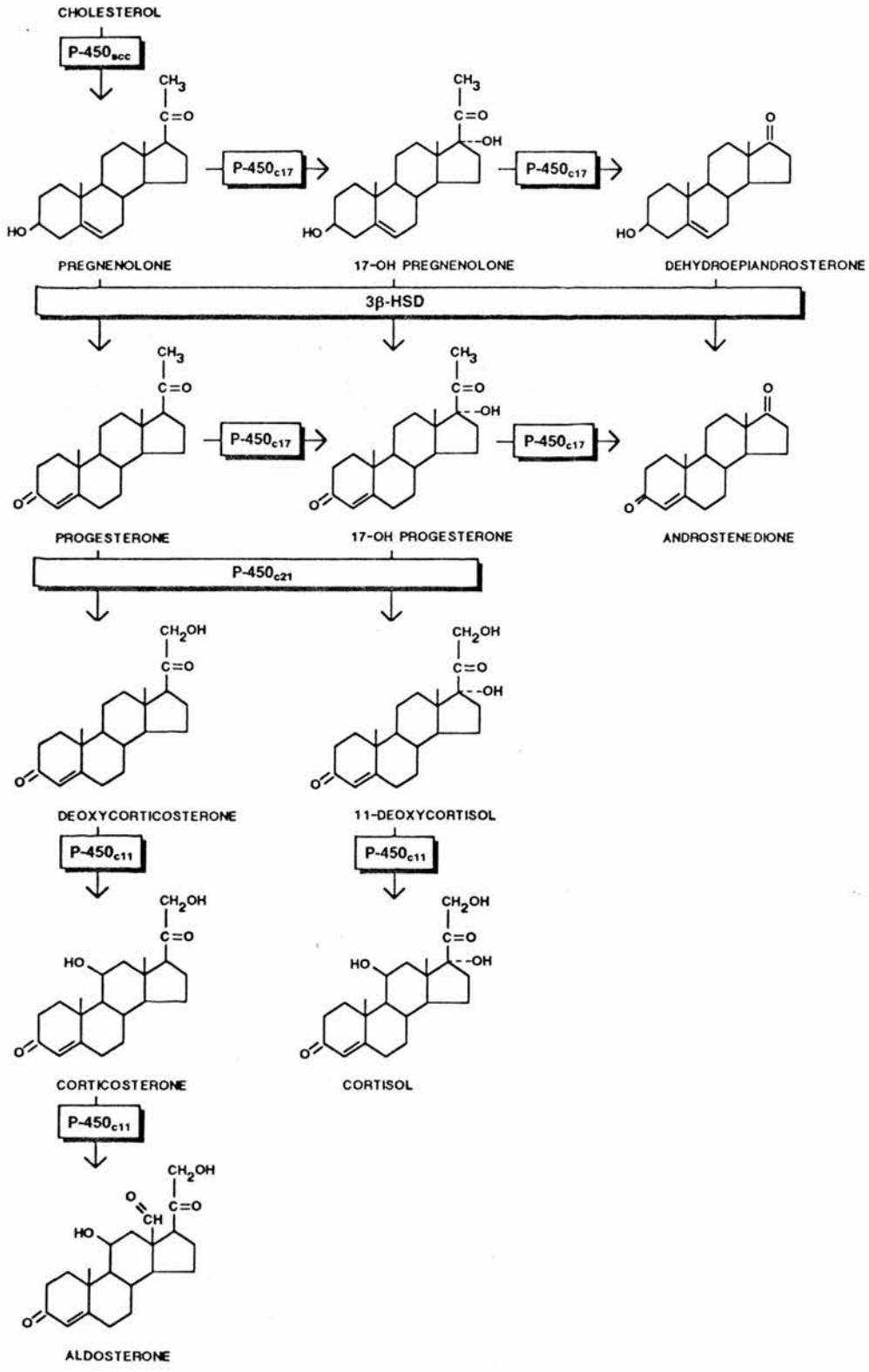



**Figure 1.2:**

Steroid biosynthetic pathways in the adrenal cortex. The pathways for mineralocorticoids, glucocorticoids and adrenal androgens are shown. 3ß-HSD=3ß-hydroxysteroid dehydrogenase. Taken from Orth et al., 1991.

C21 steroids possess mineralocorticoid and glucocorticoid activities with only small structural differences determining which activity predominates (Fig. 1.2). Mineralocorticoids are synthesised in the zona glomerulosa from deoxycorticosterone, which is converted to aldosterone in three steps, by the action of a single enzyme - P-450$_{c11}$ (aldosterone synthase). A related but separate gene product (11ß-hydroxylase) is also involved in the synthesis of glucocorticoids in the zona fasiculata (John et al., 1985; Chua et al., 1987).

C19 steroids which possess weak androgenic activity are also produced by the adrenal cortex. In fact dehydroepiandrosterone (DHEA) and its sulphate DHEAS are the most abundant products of the adrenal cortex. They are formed from 17α-hydroxypregnenolone and 17α-progesterone (Fig. 1.2) by the action of P-450c17. The products of this reaction, dehydroepiandrosterone and androstenedione are subsequently converted to testosterone in the periphery; adrenal testosterone synthesis is minimal.

Removal of a further carbon atom (C19) by the microsomal enzyme P-450 aromatase produces C18 compounds with weak oestrogenic properties. Only small amounts of oestrogens are produced from the adrenal glands, but DHEA, DHEAS and androstenedione are substrates for oestrogen production by peripheral tissues such as adipose tissue which contain P-450 aromatase activity (Nimrod & Ryan, 1975).

### 1.1.3 Steroid Release and Circulation

Adrenal steroid hormones are released immediately following synthesis, with only small amounts stored in the gland. Circulating steroid hormones are largely bound to plasma proteins. The major binding proteins are corticosteroid binding globulin (CBG), testosterone binding globulin (also known as sex steroid binding globulin) and albumin. 3-4% of plasma cortisol and corticosterone typically circulates 'free' in the absence of binding globulins; approximately 90% cortisol and 80% corticosterone is found associated with CBG, with remaining glucocorticoids normally associated with albumin (Dunn et al., 1981). In contrast, aldosterone is only weakly associated with CBG (20%) and albumin (40%), the rest being non-protein bound or 'free'.

### 1.1.4 Regulation of Glucocorticoid Secretion

Glucocorticoid secretion is regulated by interactions between hormones of the hypothalamus, the pituitary, and the adrenal glands, and by neural stimuli. Neural stimuli from the brain in response to stress for example, cause release of corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) from paraventricular parvicellular neurones into the hypophyseal portal blood. These hormones are carried in the portal

vessels to the anterior pituitary where they stimulate adrenal corticotrophic hormone (ACTH) secretion into the systemic blood. ACTH acts on the adrenal cortex to stimulate synthesis and release of glucocorticoids from the zona fasiculata and zona glomerulosa. Adrenal activity is subsequently "down-regulated" by the feedback inhibitory actions of glucocorticoids which inhibit CRH and AVP release from the hypothalamus, and ACTH release from the pituitary in a negative feedback mechanism. These regulatory systems are depicted in Fig. 1.3, and reviewed in Orth et al. (1991).

#### 1.1.4.1 *ACTH*

ACTH is synthesised in the anterior pituitary from a large precursor, pro-opiomelanocortin (POMC) (Eipper & Mains, 1980). ACTH binds to selective cell-surface receptors resulting in activation of adenylate cyclase, thus increasing levels of cyclic AMP (cAMP) which in turn activates cAMP-dependent protein kinase (protein kinase A) and phosphorylation of several proteins ultimately resulting in stimulated steroidogenesis. The acute phase of ACTH action (within minutes) involves an increase in the conversion of cholesterol to pregnenolone (Simpson & Waterman, 1988). There is also a chronic phase of ACTH action occurring within hours to days of secretion, which involves increased synthesis of most of the enzymes involved in the biosynthesis of corticosteroids, including all of the P-450 enzymes involved (Simpson & Waterman, 1988). ACTH also induces synthesis of other proteins involved in steroidogenesis such as (i) the low density lipoprotein (LDL) receptor which is required for the uptake of circulating cholesterol, (ii) adrenodoxin, a protein in the electron transport system involved in electron transfer by the P-450 enzymes and (iii) sterol carrier protein 2 which is required for the transfer of cholesterol from intracellular lipid stores to mitochondria.

In addition to a regulatory role in corticosteroid biosynthesis, ACTH is involved in the maintenance of adrenal weight. Thus, supraphysiological ACTH administration leads to adrenal hypertrophy and hyperplasia, while the absence of ACTH results in adrenal atrophy (Gill, 1972).

#### 1.1.4.2 *Regulation of ACTH Secretion*

Although CRH and AVP are the most important physiological regulators of ACTH secretion, several other hormones such as catecholamines, angiotensin II, serotonin, oxytocin, atrial natriuretic factor (ANF), cholesystokinin, vasoactive intestinal peptide, PHI-27 (a gastrointestinal and hypothalamic peptide) and gastrin-releasing peptide have also been implicated (reviewed in Antoni, 1986).

AVP that participates in the regulation of ACTH release is produced two groups of neurones in the hypothalamus; the parvicellular cells of the paraventricular nucleus which also produce CRH, and the magnocellular cells in the supraoptic and paraventricular

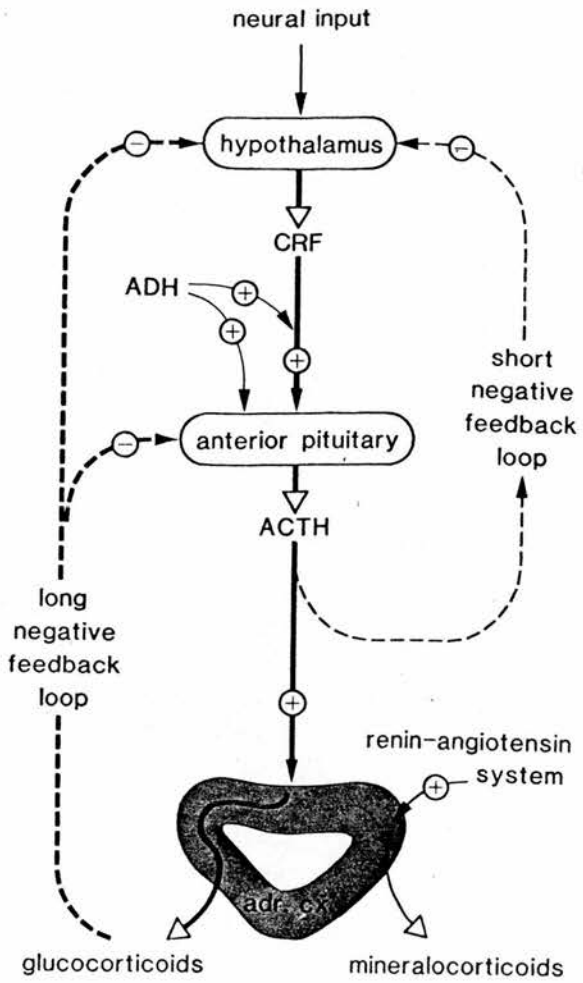



**Figure 1.3:**

Control of glucocorticoid synthesis and secretion; the hypothalamic-pituitary-adrenal axis. + = stimulation; - = inhibition.

nuclei. AVP binds to selective receptors resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate and mobilisation of intracellular calcium stores. AVP control of ACTH secretion is reviewed in Antoni (1993).

CRH is a 41 amino acid peptide that is probably the major regulator of ACTH secretion. Similarly to ACTH, CRH mediates its effects by binding to selective cell-surface receptors which promote activation of adenylate cyclase and cAMP- dependent protein kinase A. Within seconds, this results in ACTH secretion from the anterior pituitary corticotropes, and subsequently increased POMC gene transcription.

#### 1.1.4.3 *Other Regulatory Systems*

Glucocorticoid secretion is potentially regulated by three control and response systems which may operate simultaneously. (i) The hypothalamic-pituitary-adrenal axis in humans and other species, is regulated by a circadian ("about a day") rhythm. The circadian rhythm of ACTH and glucocorticoid secretion is modified by light and sleep patterns, such that both ACTH and glucocorticoids reach a maximum in the last few hours before and after wakening, and then decline throughout the morning and are minimal in the evening. The mechanisms responsible for the circadian rhythm are unclear; a diurnal rhythm of CRH release has been reported by some (Watabe et al., 1987), but not by others (Cunnah et al., 1987) suggesting that other factors must be involved in maintenance of the circadian rhythm of ACTH and glucocorticoid secretion. (ii) Stress activates the hypothalamic-pituitary-adrenal axis. Physical and psychological stressors such as exercise, trauma, surgery, cold exposure, fear and depression all stimulate the hypothalamus to release ACTH secretagogues such as CRF and AVP. (iii) Glucocorticoid feedback occurs both at the level of the hypothalamus, and the pituitary. In the anterior pituitary, glucocorticoids inhibit ACTH secretion and POMC gene transcription resulting in reduced POMC synthesis (Lundbland & Roberts, 1988). Glucocorticoids also decrease CRF and AVP mRNA and peptide levels in the paraventricular nucleus of the hypothalamus.

### 1.1.5 Regulation of Mineralocorticoid Secretion

Aldosterone secretion is largely controlled by the renin-angiotensin system and potassium ions. In addition, ACTH and other POMC-derived peptides, sodium ions, AVP, dopamine, ANF, serotonin and somatostatin may have minor regulatory roles (Quinn & Williams, 1988).

1.1.5.1 *The Renin-Angiotensin-System*

Renin is an enzyme synthesised by the juxtaglomerular cells of the renal cortex, which catalyses the conversion of liver synthesised angiotensinogen to angiotensin I (Fig. 1.4) (Goodfriend et al., 1984). Angiotensin I is rapidly converted to angiotensin II by the angiotensin converting enzyme (ACE) predominantly in the lungs and vasculature. Angiotensin II and in addition angiotensin III, (produced by cleavage of the amino terminal asparagine from angiotensin II) are responsible for stimulating aldosterone secretion (Goodfriend et al., 1984). In contrast, angiotensin I has no known biological action. The actions of angiotensins II and III on the zona glomerulosa are mediated by cell surface receptors linked to activation of phospholipase C via one or more GTP-binding proteins. Release of intracellular and extracellular calcium ions through the phosphoinositol pathway then promotes conversion of cholesterol to pregnenolone, and corticosterone to aldosterone (Kramer et al., 1980).

Renin secretion and release from the kidney is controlled by renal arterial blood pressure and sodium levels in the renal tubular fluid (Gibbons et al., 1984). Thus factors which increase blood pressure such as high salt intake will result in a decrease renin secretion, while factors which decrease blood pressure such as haemorrhage and dehydration will increase renin secretion. In addition, angiotensin II inhibits renin release by negative feedback.

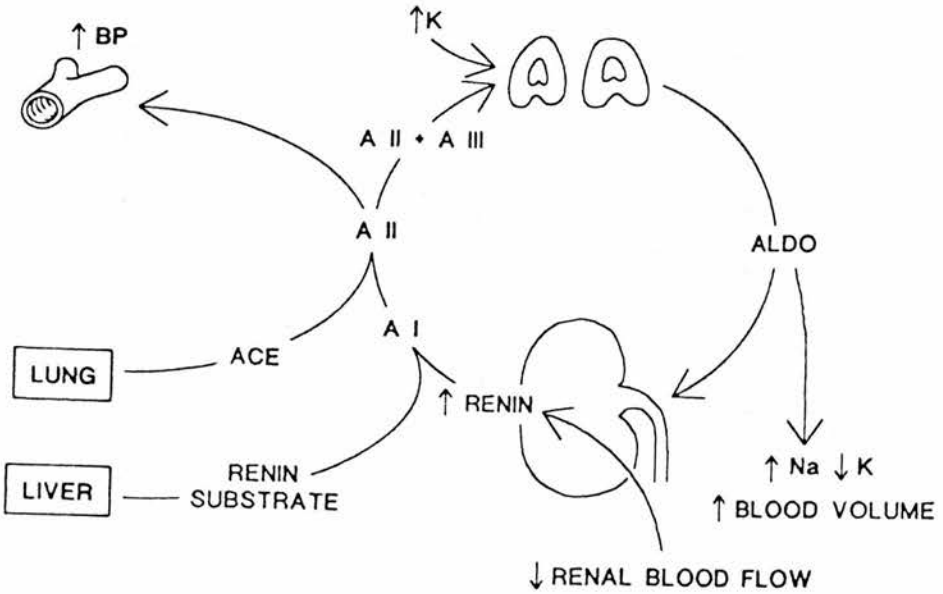



**Figure 1.4:**

Control of aldosterone secretion by the renin-angiotensin-aldosterone pathway. ACE = angiotensin converting enzyme; AI, AII, AIII = angiotensins I, II and III.

1.1.5.2 *Potassium*

Regulation of aldosterone secretion by potassium ions also involves calcium ions as a second messenger system. Potassium directly increases aldosterone secretion from the adrenal cortex by depolarizing the plasma membrane which activates voltage-dependent calcium channels, resulting in an influx of extracellular calcium (Quinn & Williams, 1988) which subsequently increases the activity of steroidogenic enzymes, and thus promotes conversion of cholesterol to pregnenolone and corticosterone to aldosterone in the same way as angiotnesin II (McKenna et al., 1978).

1.1.6 Effects of Glucocorticoids

Glucocorticoids have profound effects on development and in the adult, both peripherally and centrally. For example, glucocorticoids regulate homeostasis, affect mood and behaviour, are involved in the inflammatory response, and have profound effects in development; in fact excess glucocorticoid exposure during development leads to growth retardation, and possibly increases the risk of high blood pressure and ischaemic heart disease mortality (section 1.3.6.1). A deficiency in corticosteroid production leads to Addison's disease which is characterised by muscular weakness, low blood pressure, depression and weight loss. Conversely, Cushing's syndrome occurs as a result of overproduction of glucocorticoids which may lead to hypertension, poor wound healing, and redistribution of body fat. The effects of glucocorticoids discussed briefly below, are reviewed in detail by Orth et al. (1991).

1.1.6.1 *Fuel Metabolism*

Glucocorticoids play an important role in energy production from proteins, carbohydrates and fats. Glucocorticoids increase gluconeogenesis in the liver by increasing the supply of gluconeogenic amino acids from breakdown of muscle proteins, and by activation of gluconeogenic enzymes such as glucose-6-phosphate (G-6-P) and phosphoenol pyruvate carboxykinase (PEPCK) (Exton, 1979). In conjunction with increased gluconeogensis, glucocorticoids also inhibit glucose uptake and peripheral tissue glucose utilisation in part by direct inhibition of glucose transport into cells (Munk, 1962). Glucocorticoids increase glycogen storage in the liver which may be due to insulin secretion in response to the increase in blood sugar. There is also increased protein breakdown and decreased protein synthesis, particularly in muscle in response to glucocorticoids. Glucocorticoids acutely activate lipolysis in adipose tissue (Fain, 1979), and lead to redistribution of body fat in situations of glucocorticoid excess possibly in response to hyperinsulinaemia (Hausberger, 1958). Finally glucocorticoids appear to have a permissive role in mediating the effects of other gluconeogenic enzymes such as

glucagon and catecholamines which are ineffective in the absence of glucocorticoids. Thus glucocorticoids affect fat metabolism by enhancing the sensitivity of lipolysis to catecholamines (Exton, 1979).

1.1.6.2 *Immunological Function and Inflammatory Processes*

When given therapeutically, glucocorticoids have powerful anti-inflammatory and immunosupressive effects (Graham & Tucker, 1984). Local inflammatory responses are mediated by movement of cells and fluid from the intravascular compartment to the site of inflammation. Glucocorticoids inhibit this process (Zweifach et al., 1953) possibly by inhibiting prostaglandin synthesis (Hong & Levine, 1976). Glucocorticoids also inhibit the actions of inflammatory mediators such as histamine which is a potent vasoactive agent (Facui, 1979).

Probably the major effect of glucocorticoids on immune cells is to influence their movement to and from the peripheral circulation. Thus glucocorticoids appear to deplete T-cells and B-cells by redistribution from the intravascular compartment to the spleen, lymph nodes and bone marrow (Facui & Dale, 1974; Yu et al., 1974). Glucocorticoids also have immunosupressive effects on T-cells and B-cells by inhibiting their proliferation (Gillis et al., 1979; Cupps et al., 1985). In addition, glucocorticoids inhibit the phagocytic and cytotoxic functions of macrophages (Rinehart et al., 1982).

1.1.6.3 *Musculoskeletal and Connective Tissues*

Chronic glucocorticoid excess causes osteoporosis implicating direct steroid effects on bone cells. Glucocorticoids inhibit osteoblast function and increase osteoclast number, leading to a reduction in new bone formation (Hahn et al., 1979). Glucocorticoids also appear to mediate indirect effects on bone by inhibiting intestinal calcium reabsorption (Hahn et al., 1979; Wajchenberg et al., 1969).

Fibroblast proliferation is inhibited by glucocorticoids. Glucocorticoids also inhibit synthesis of the extracellular matrix components collagen and hyaluronidase which collectively result in impaired wound healing and fragile connective tissues (Leibovich & Ross, 1975).

1.1.6.4 *Fluid and Electrolyte Homeostasis*

Evidence of glucocorticoid modulation of blood pressure comes mainly from patients with glucocorticoids excess or deficiency. Patients with glucocorticoid excess usually suffer from hypertension, often without increased levels of mineralocorticoids, kaliuresis or suppressed plasma renin activity (Krakoff et al., 1975; Saruta et al., 1986). However whether glucocorticoids exert a direct mineralocorticoid response is unclear. Other glucocorticoid effects have been implicated in the modulation of blood pressure. For

example, glucocorticoids induce hepatic production of angiotensinogen, but the mechanism is unclear, as is the issue of whether increased circulating angiotensinogen levels leads to increased angiotensin II production. In addition, decreased levels of the vasodilators prostaglandin $E_2$ and kallikrein are found in the urine of animals and humans with glucocorticoid excess (Saruta et al., 1986; Handa et al., 1983). Thus the basis for hypertension in patients with glucocorticoid excess is poorly understood.

A decrease in free water clearance in patients with glucocorticoid deficiency is associated with increased plasma AVP concentrations (Slessor, 1951; Garrod & Burston, 1952). In addition, glucocorticoid receptors are present in AVP producing cells of the parvicellular division of the paraventricular hypothalamus (Uht et al., 1988), and glucocorticoid deficiency leads to increased AVP mRNA levels in the paraventricular nucleus (Widmaier et al., 1988). In addition, increased circulating AVP levels may play a role in maintaining blood pressure in adrenal insufficiency (Schwartz et al., 1983).

Animals which have had their adrenal glands removed apart from the adrenal capsule and zona glomerulosa appear unable to secrete excess salt (Gaunt et al., 1967; 1968). Glucocorticoid replacement reverses this effect by inducing the synthesis and secretion of ANF from cardiac myocytes (Garcia et al., 1985). The ANF gene contains putative glucocorticoid response elements on its regulatory region (Greenberg et al., 1984; Seidman et al., 1984; Argentin et al., 1985) suggesting that the effects of glucocorticoids may be direct.

#### 1.1.6.5 *Neuropsychiatric and Behavioural Effects*

Glucocorticoids affect mood and sleep patterns in humans (McEwen, 1979). The duration of rapid eye movement sleep is decreased in patients with Cushing's Syndrome (Kreiger, 1972), and in normal subjects treated with exogenous glucocorticoids (Gallin et al., 1972). Evidence for glucocorticoid-induced changes in mood also come largely from clinical observations. Approximately 90% of patients with Cushing's Syndrome suffer from psychological disturbances including depression (Cohen, 1980). In addition patients with adrenal insufficiency may also suffer from psychological disturbances with depression, apathy and lethargy being the most common. The mechanisms of action of these behavioural effects of glucocorticoids are unknown.

#### 1.1.6.6 *Gastrointestinal Effects*

Although the colon contains mineralocorticoid receptors which may mediate ion transport in the colon, there is also evidence that glucocorticoids act through glucocorticoid receptors to induce sodium transport. This has been shown through the use of specific glucocorticoid receptor analogues to demonstrate a saturable effect on sodium transport (Bastl, 1987). In addition, spironolactone which antagonises

mineralocorticoid receptors has no effect on the sodium transport response to low dose dexamethasone (a synthetic glucocorticoid, selective for glucocorticoid receptors) treatment (Bastl, 1988).

1.1.6.7 *Developmental Effects*

Glucocorticoids have many and profound effects on development. For example, glucocorticoids regulate organ development and maturation, and of particular clinical importance are the effects of glucocorticoids in pre- and perinatal lung maturation. Glucocorticoids stimulate surfactant production by type II pneumocytes, a normal developmental process which can be accelerated by exogenous glucocorticoids (Ballard, 1987). Glucocorticoids also cause morphological changes in type II cells (Liley et al., 1987), induce enzymes involved in phospholipid biosynthesis and regulate transcription of the gene for the major surfactant protein (SP-A) (Odom et al., 1988; Boggaram & Mendelson, 1988). Therefore threat of premature labour is sometimes treated with dexamethasone to promote surfactant synthesis and release in the foetal lungs.

High glucocorticoid exposure during development may lead to retardation of foetal growth (Reinisch et al., 1978) and may increase the risk of developing hypertension and ischaemic heart disease mortality in later life (Barker et al., 1989a; 1989b; Whincup et al., 1989; Barker et al., 1990). Foetal exposure to glucocorticoids may also affect processes which involve cell death presumably due to direct inhibitory effects on bone and connective tissue (section 1.1.6.3). High glucocorticoid exposure may therefore result in incidences of cleft palate and polycystic kidney.

In the developing nervous system, glucocorticoids regulate the differentiation of neural crest epithelium cells into chromaffin cells. Neural crest cells are precursors of several differentiated cell types including autonomic ganglion cells and adrenomedullary cells. It is under the influence of glucocorticoids that neural crest precursor cells, which invade the embryonic adrenal gland, cease to express neurone-specific gene products such as neurofilaments, and begin to achieve adrenal chromaffin cell morphology (Fenderoff et al., 1988). The mechanisms by which glucocorticoids mediate this differential process is not known.

1.1.7 Effects of Mineralocorticoids

Aldosterone is the most important and most potent endogenous mineralocorticoid whose physiological role is to promote sodium reabsorption and potassium loss from urine in kidney distal tubules and cortical collecting tubules, from gastrointestinal contents in the colon, from saliva in the salivary glands, and from sweat (reviewed in Morris, 1981). Probably the most important of these sodium retaining functions is that in

kidney tubules, since electrolyte secretion and therefore extracellular volume and blood pressure are generally controlled by this process. Aldosterone has almost the same effects on sweat glands and salivary glands that it has on renal distal tubules. Both glands contain a primary secretion that contains large quantities of sodium chloride, but much of the sodium chloride is reabsorbed, while potassium and bicarbonate ions are secreted. The effect on sweat glands is important to conserve body salt in hot environments, and the effect on salivary glands is necessary to conserve salts when excessive amounts of saliva are lost.

Aldosterone may also act on non-epithelial tissues. For example there is evidence for aldosterone-selective actions in brain (McEwen et al., 1986a). Aldosterone infused intracerebroventricularly (icv) at low concentrations has effects on salt seeking and ingestion that are not mimicked or blocked by equal or higher amounts of corticosterone, but are largely blocked by specific aldosterone antagonists (McEwen et al., 1986a). In addition, there are recent reports of aldosterone actions on circulating mononuclear leukocytes (Wehling et al., 1987). Nanomolar concentrations of aldosterone block the ion flux that otherwise occurs during the process of cell preparation by Ficoll-Hyapaque centrifugation. This effect is neither blocked nor mimicked by cortisol. Thus as well as the traditional epithelial actions of aldosterone in kidney distal tubules, sweat glands and salivary glands, there may also be non-epithelial effects of aldosterone.

## 1.2 Steroid Receptors

Steroid hormones classically elicit their effects by binding to steroid hormone receptors to activate or transform the receptors, which act as nuclear transcription factors that modulate cell function by either activating or repressing target genes or gene networks (reviewed by Yamamoto, 1985). Activated receptors can then specifically bind to DNA sequences called hormone response elements (HREs) which are usually found in the 5' regulatory region of target genes. The steroid receptor-steroid HRE interaction then acts to either induce or repress target gene transcription.

### 1.2.1 Steroid Hormone Receptor Superfamily

The realisation that steroid hormone receptors belonged to a highly homologous family became apparent when the various human and rat steroid receptors were cloned (reviewed by Green & Chambon, 1988; Evans, 1988; Berg, 1989; Beato, 1989; O'Malley, 1990; King, 1992), and comparative analyses were performed. It is now known that the steroid receptor superfamily includes glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), oestrogen receptor (ER) and androgen receptor (AR). The existence of related nuclear receptors that respond to ligands other than steroid hormones was demonstrated by the finding that the DNA binding domain of GR was remarkably similar to the viral oncogene *v-erbA* and subsequent identification of *c-erbA* as the thyroid hormone receptor (Weinberger et al., 1986). Molecular cloning studies have since demonstrated three more thyroid receptors (ie now $TR_{\alpha 1}$, $c\text{-}erbA_{\alpha 2}$, $TR_{\beta 1}$, $TR_{\beta 2}$), three retinoic acid receptors ($RAR_{\alpha}$, $RAR_{\beta}$, $RAR_{\gamma}$), three retinoid X receptors ($RXR_{\alpha}$, $RXR_{\beta}$, $RXR_{\gamma}$) and vitamin D receptor (VDR) which are all superfamily members. In addition, there appear to be several other superfamily members for which no known activating ligand has been identified. For this reason, these members are known as orphan receptors (reviewed by O'Malley, 1990; King, 1992). Orphan receptors include at least two chicken ovalbumin upstream promoter transcription factor (COUP-TF), two oestrogen receptor related proteins called ERR1 and ERR2, peroxisome proliferator activated receptor (PPAR), the nerve growth factor-induced NGFI-B protein, the hepatic nuclear factor HNF-4 and steroidogenic factor (SF1). It has been predicted that the ligands for these orphan receptors may be indigenous to the cells in which particular orphans are found (O'Malley, 1990). Alternatively, phosphorylation may regulate the function of orphan receptors (O'Malley & Conneely, 1992). Finally, the steroid hormone receptor superfamily also appears to incorporate a variety of steroid hormone related proteins which regulate developmental pathways in invertebrates. The

invertebrate proteins include the ecdysone receptor, the seven up protein and Fushi-tarazu (FTZ-F1) protein (Parker, 1993).

### 1.2.2 Functional Domains of Steroid Receptors

Analysis of steroid receptor structure is essential for understanding how steroid receptors mediate transcriptional control of target genes. Before steroid receptors were cloned, biochemical and immunological analysis of purified rat liver GR provided the first evidence of a domain structure for steroid receptors. Thus it was shown that GR is composed of three distinct domains, two of which function independently of each other and contain information for DNA binding and steroid hormone binding, while the third N-terminal domain contains a major epitope for immunoreactivity (Carlstedt-Duke et al., 1982). The molecular cloning of GR and other receptors permitted functional and structural comparisons of steroid receptors. It is now known that the ligand binding domain is C-terminally located while the DNA binding domain is located towards the middle of the receptor (Fig. 1.5) (reviewed by Evans, 1988; Green & Chambon, 1988; Beato, 1989; O'Malley, 1990; King, 1992). Throughout all steroid receptors these two domains, and in particular the DNA binding domain, share high degrees of homology. In contrast, the N-terminal domain is not conserved.

#### 1.2.2.1 *The Ligand Binding Domain*

The first step in the cascade of events leading to induction or repression of target gene transcription by steroid hormones is binding of the hormone to the carboxy terminal ligand binding domain of the receptor. Deletion of the steroid binding domain results in constitutive activation of steroid receptors in the absence of hormone binding (Miesfeld et al., 1987). Thus it appears that the hormone binding domain acts as a repressor of steroid receptor activation in the absence of hormone and that this repression represents an intrinsic property of the receptor. Repression of steroid activation appears to be mediated by the association of steroid receptors with the 90kD heat shock protein (hsp90) in the cytoplasm of target cells. Ligand binding leads to the dissociation of hsp90, thus relieving repression of steroid receptor activation (reviewed for GR by Muller & Renkawitz, 1991; Danielson, 1991).

The cellular localisation of unoccupied steroid receptors has been controversial for many years, with some data suggesting predominance in the cytoplasm and others indicating nuclear localisation (reviewed by Walters, 1985; Jenson, 1990). However recent work has shown that GR and PR are shuttled between the nucleus and the cytoplasm (DeFranco et al., 1991; Guiochon-Mantel et al., 1991) suggesting that

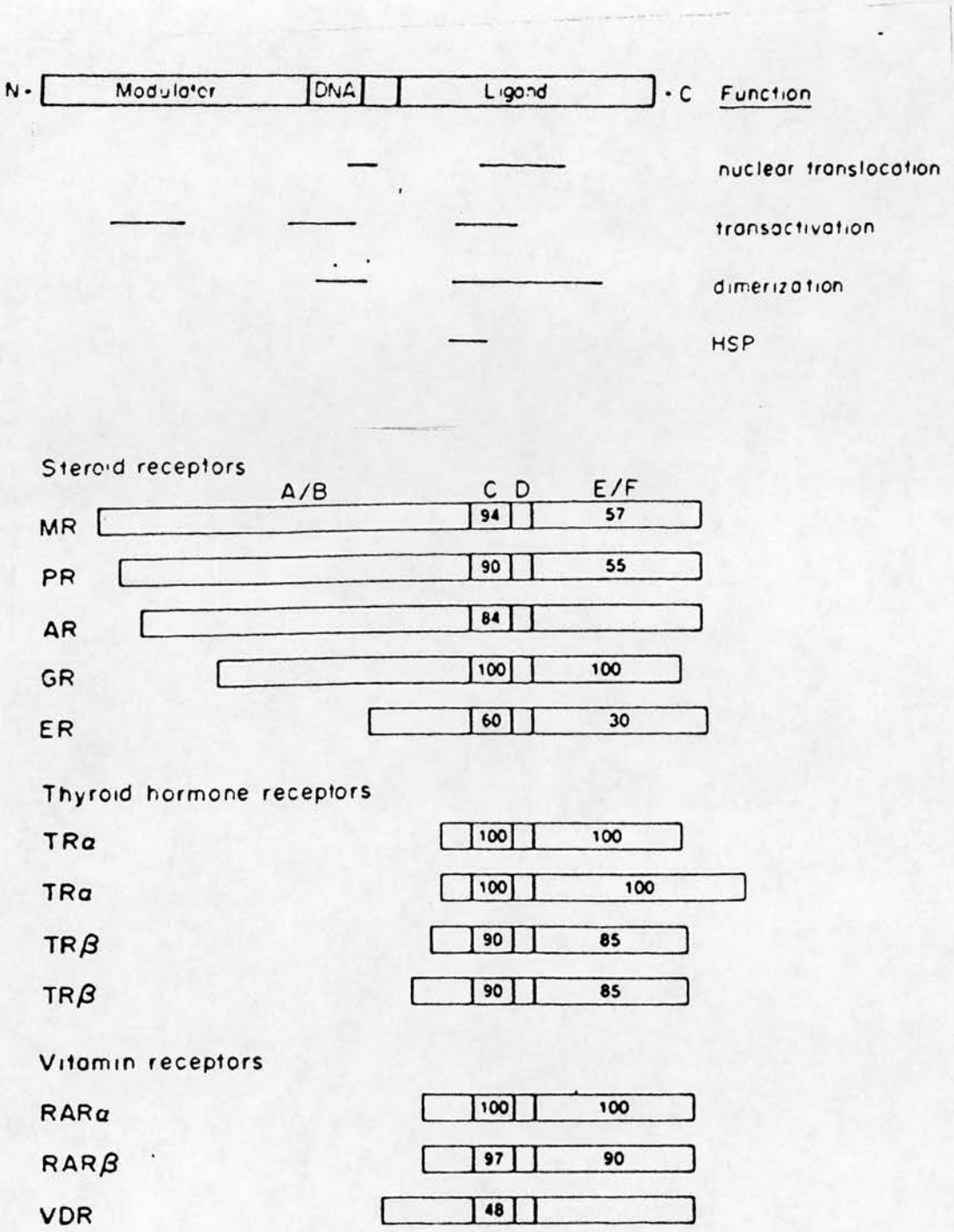



**Figure 1.5:**

Homologies between members of the steroid hormone receptor superfamily. The numbers between E/F, the steroid binding domain and C, the DNA binding domain indicate the percentage homology. Members of the family have been arranged within groups with GR, $TR_\alpha$ and $RAR_\alpha$ arbitrarily assigned 100%. For comparison of DNA binding domain between groups, if GR=100%, then TRα=44% and RARα=46%. Adapted from King, 1991.

measurements of cytoplasmic and nuclear receptor localisation are a reflection of an equilibrium between the two locations.

Two domains of rat GR have been identified that are involved in translocation of activated steroid receptors to the nucleus (Picard & Yamamoto, 1987). One sequence is located in the ligand binding domain, and the other in the hinge region of the DNA and ligand binding domains. Both sequences are necessary to direct the hormone-receptor complex to the nucleus.

1.2.2.2 *Receptor Dimerisation*

Steroid receptors bind to target DNA as dimers, and this also appears to be a function of the carboxy terminal steroid binding domain. The steroid hormone receptor superfamily can be divided into subfamilies dependent on dimerisation properties. Thus steroid hormone receptors such as MR and GR appear to function as homodimers. In contrast, although receptors such as TR, RAR and VDR have been shown to bind to DNA as homodimers, this binding was relatively weak. However it was shown that binding could be enhanced by proteins in nuclear extracts of various types. The protein responsible for enhanced binding has now been cloned, and was found to be another member of the nuclear hormone receptor superfamily, RXR (Yu et al., 1991; Leid et al., 1992). RXR$\alpha$ and RXR$\beta$ have been shown to stimulate binding of TR, RAR, VDR, COUP-TF and PPAR to their respective DNA binding sites suggesting that these hormone receptors are most likely to function as heterodimers (Kliewer et al., 1992; Zhang et al., 1992; Bugge et al., 1992). Finally, it is likely that superfamily members such as NGFI-B and FTZ-F1 proteins will function as monomers (Parker, 1993).

1.2.2.3 *Target Gene Recognition*

The DNA binding domain is sufficient for specific binding of steroid receptors to target genes *in vivo* and *in vitro*. However, high affinity binding to DNA requires additional information encoded within the N and C-terminal domains of the receptor (Danielson et al., 1987). The DNA binding domain is highly basic and contains a number of highly conserved cysteine residues, eight of which are involved in the formation of two zinc fingers (Evans & Hollenberg, 1988; Frankel & Pabo, 1988). In the case of GR, the zinc finger consists of two pairs of cysteine residues co-ordinated with a zinc atom (Hard et al., 1990). Two zinc finger motifs are present in the DNA binding domain, and the zinc atom is essential for DNA-binding. The first (N-terminal) zinc finger is involved in the distinction between glucocorticoid response element (GRE) and ERE. Three residues at the C-terminus of the first finger and in the interfinger region are sufficient for specific GRE recognition. Mutational analysis of the second zinc finger has demonstrated that it is also required for stabilisation of the interaction (Mader et al.,

1989; Danielson et al., 1989). It has recently been shown that the second zinc finger is involved in distinction between GRE and ERE or TRE (thyroid response element). Structural nuclear magnetic resonance data using the GR DNA binding domain expressed in *E. coli* has confirmed the existence of zinc fingers, but the region contacting the DNA was shown to be an α-helical structure located between the two zinc fingers (Hard et al., 1990). The first chrystallographic data for the structure of GR DNA-binding domain complexed with DNA has also recently been obtained (Luisi et al., 1991). The structure revealed that amino acids in the two zinc fingers which are important for target gene recognition, are found in a helical region which makes contact with the major groove of DNA. It was also revealed that residues in the second zinc finger are involved in dimerization of the DNA binding domain (Luisi et al., 1991).

One of the most remarkable features of steroid receptors is that they bind to DNA response elements consisting of only two distinct sequence motifs related to either AGAACA or AGGTCA. DNA binding specificity appears to be determined by the orientation and relative spacing of these motifs. For example, AR, GR, MR and PR all bind to a steroid response element known as the glucocorticoid response element (GRE) which is an inverted repeat of AGAACA separated by three nucleotides (Table 1.1). Other steroid receptors are capable of binding to varients of the AGGTCA sequence as inverted or direct repeats, although direct repeats are most common in genes. COUP-TF and RAR bind to direct repeats in a rather promiscuous manner (Table 1.1). Thus RAR binds to direct repeats separated by 1, 2 or 5 nucleotides (Umesono et al., 1991; Durand et al., 1992), while COUP-TF is able to bind to direct repeats with spacings of between 1 and 6 nucleotides (Cooney et al., 1992). Thus while the specificity of hormonal responses is determined in part by a recognition code, additional factors must be involved to confer specificity if cross-talk between hormonal responses is to be avoided.

The members of the steroid hormone superfamily which have been proposed to bind to DNA in target genes as monomers (eg NGFI-B, FTZ-F1), appear to bind to a single AGGTCA motif proceeded by three adenine nucleotides in the case of NGFI-B (Wilson et al., 1991) or PyCA for FTZ-F1 (Ueda et al., 1992).

#### 1.2.2.4 *Transcriptional Regulation*

The hormone binding domain and the amino-terminal domain of steroid receptors appear to be important for efficient transcriptional activation. Several models have been developed to explain the mechanism by which transcriptional activation is regulated. The first was initially developed for prokaryotic promoters (Ptashne, 1986, 1988), and suggests that interaction of the DNA bound hormone receptor complex with a component(s) of the transcriptional machinery of the cell to form a transcription activation complex may be involved in transcriptional regulation. Alternatively it has

| DNA binding sites | | Receptors |
|---|---|---|
| Steroid response elements | | |
| AGAACA nnn TGTTCT | GRE | AR, GR, MR, PR |
| AGGTCA nnn TGACCT | ERE | ER |
| Direct repeats | | |
| AGGTCA n AGGTCA | DR-1 | RXR-RXR, PPAR-RXR, RAR-RXR, COUP-TF-RXR |
| AGGTCA nn AGGTCA | DR-2 | RAR-RXR |
| AGGTCA nnn AGGTCA | DR-3 | VDR-RXR |
| AGGTCA nnnn AGGTCA | DR-4 | TR-RXR |
| AGGTCA nnnnn AGGTCA | DR-5 | RAR-RXR |

**Table 1.1:**
DNA-binding sites for nuclear receptors. GRE, glucocorticoid response element; ERE, oestrogen response element; n, any nucleotide; DR-n, number of nucleotides between direct repeats. Taken from Umesono et al., 1991.

been proposed that DNA bound hormone receptor complexes may realign nucleosomes to allow transcription factor recognition of and binding to previously inaccessible regions of the target gene promoter (Archer et al., 1991). It is also possible that a combination of these two mechanisms may be used to regulate transcriptional activation.

In addition to their role as transcriptional activators, many nuclear receptors have been shown to repress gene transcription. Several alternative mechanisms have been suggested, including competition for common DNA-binding sites. Recently, a novel mechanism has been proposed for the glucocorticoid repression of the POMC gene (Drouin et al., 1993). The POMC gene contains a negative GRE (nGRE), GGAAGGTCAGGTCCA) which has been shown to bind three GR molecules apparently formed by binding of a GR homodimer followed by binding of a GR monomer on the opposite side of the double helix. It is thought that GR inhibition of transcription may be mediated by interference with the activity of other transcription factors or the transcriptional machinery.

1.2.2.5 *Hormone Specificity and Crosstalk With Other Signalling Pathways*

It is now known that members of the steroid hormone receptor superfamily can interact with members of the AP-1 family of transcription factors to stimulate or repress gene transcription (Miner & Yamamoto, 1991; 1992). It has been shown that the ability of GR to stimulate proliferin gene transcription is enhanced by c-jun homodimers, but repressed by c-jun : c-Fos heterodimers. The effects are mediated by an element related to but distinct from, a GRE which binds GRE and AP-1, called a composite GRE. These effects cannot be reproduced by replacing GR with MR or c-Fos with Fra-1. Thus one mechanism for generating steroid-specific responses in cells expressing multiple types of receptor involves interactions with AP-1 family members.

1.2.3 Corticosteroid Receptors: How Many Types?

It is evident that MR has very high affinity for both glucocorticoids and mineralocorticoids *in vitro* (Krozowski & Funder, 1983). However *in vivo*, MR in kidney selectively bind aldosterone, whereas the physiological ligand for MR in hippocampus and heart is cortisol or corticosterone. Cloning of human MR (hMR; Arriza et al., 1987) and hGR (Hollenberg et al., 1985) cDNAs has demonstrated that the receptors are highly homologous in both the DNA binding domains and the ligand binding domains as discussed previously. Furthermore, the isolation of an MR cDNA from a rat brain library indicated that MR in kidney and brain are identical (Arriza et al., 1988; Patel et al., 1989). Thus tissue-specific differences in aldosterone and glucocorticoid activation of MR cannot be explained by receptor differences.

The hormone binding specificity of recombinant expressed hMR and hGR has been investigated by Arriza et al. (1988). A functional assay was developed where either hMR or hGR were subcloned into expression vectors and transiently transfected into CV-1 cells which have no endogenous steroid receptors. This vector is known as the trans vector. A reporter plasmid vector (the cis vector) in which a hormone-responsive promoter is linked to a structural gene encoding an easily measurable enzyme activity such as bacterial chloramphenicol acetyltransferase (CAT) or firefly luciferase, is co-transfected with the receptor expression vector. Addition of hormone to the transfected cell medium stimulates the receptor to activate transcription from the cis vector promoter. Thus CAT or luciferase activity are convenient measures of receptor-regulated gene transcription. In this case, the reporter plasmid was a chimaera of the mouse mammary tumour virus long terminal repeat (MMTV-LTR) which is sensitive to glucocorticoids, linked to the coding region of bacterial chloramphenicol acetyltransferase gene (CAT) (Fig. 1.6).

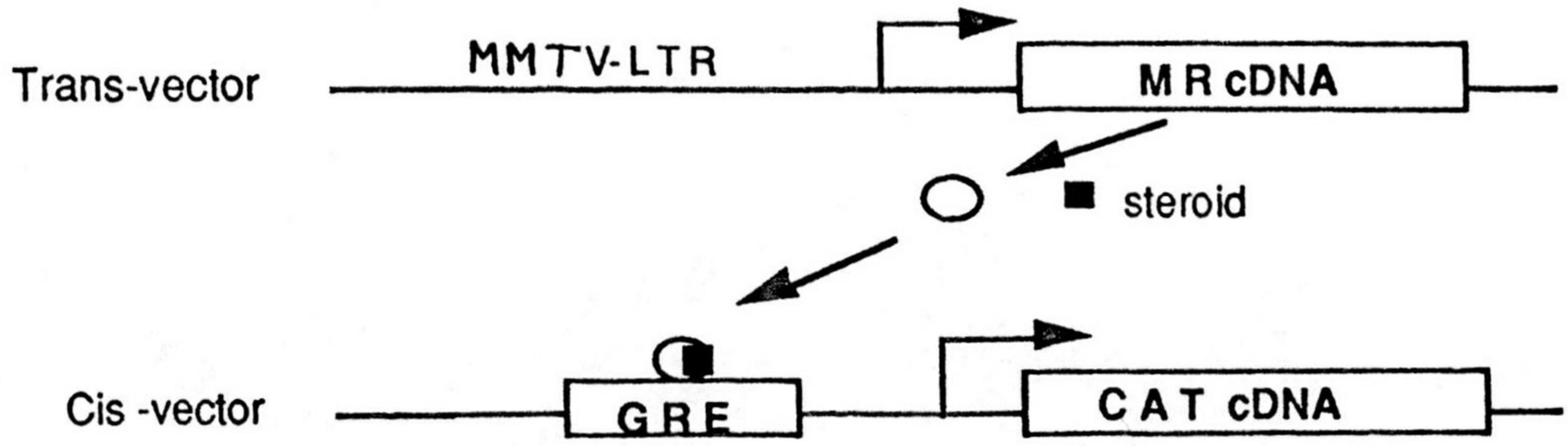



**Figure 1.6:**
The co-transfection assay. Taken from Evans & Arriza, 1989.

Cortisol and corticosterone were found to be effective at activating both MR and GR, but activated MR at 10-fold lower concentrations than GR (ie Kd for MR is 0.5nM, Kd for GR is 5nM). Thus hMR appears to have little intrinsic ability to discriminate between glucocorticoids and mineralocorticoids when recombinantly expressed in mammalian cells. As a direct result of this, it has been speculated that glucocorticoid action is determined by two receptor systems differing not only in their affinity for glucocorticoids but also in their interactions with common sets of genes (Arriza et al., 1988). MR is proposed to partially stimulate GR-responsive gene networks at low glucocorticoid levels, but maximal activation is dependent on GR responding to higher glucocorticoid levels such as those associated with the stress response for example. This proposal is particularly relevant in cells where MR and GR are coexpressed such as hippocampal neurones (Evans & Arriza, 1989).

Another question which arises however, is how do renal MR maintain aldosterone selectivity in vivo, especially in the face of a 100 to 1000-fold excess of glucocorticoids? Two studies have demonstrated that selective binding and activation of renal MR can be determined, at least in part, by the intracellular enzyme 11ß-hydroxysteroid dehydrogenase (Edwards et al., 1988; Funder et al., 1988).

## 1.3 11ß-Hydroxysteroid Dehydrogenase

### 1.3.1 Background and History

11ß-hydroxysteroid dehydrogenase (11ß-OHSD) is a microsomal enzyme which catalyses the reversible conversion of cortisol to the inactive glucocorticoid, cortisone in man, and corticosterone to the inactive 11-dehydrocorticosterone in rats (Fig. 1.7). The two activities of the enzyme are separable, with the 11ß-dehydrogenase activity oxidising the hydroxyl group at C11 to an inactive 11-keto group, while the 11ß-reductase component catalyses the reverse reaction. In 1960 (Osinski et al., 1960) first reported that 11ß-OHSD activity was dependent on the presence of NADP for 11ß-dehydrogenase activity, and NADPH for 11ß-reductase activity.

Between 1960 and 1985, 11ß-OHSD activity was demonstrated in various tissues *in vitro*. Thus 11ß-OHSD activity has been found in homogenates, subcellular fractions and cultured cells of kidney (Murphy, 1981), liver (Hurlock & Talalay, 1959; Koerner & Hellman, 1964; Bush et al., 1968), brain (Peterson et al., 1965; Grosser, 1966), lung (Koerner, 1966; Murphy, 1981; Nicholas & Lugg, 1982; Abramowitz et al., 1982; 1984), placenta (Osinski et al., 1960) and gonads (Koerner, 1966; Murphy, 1981).

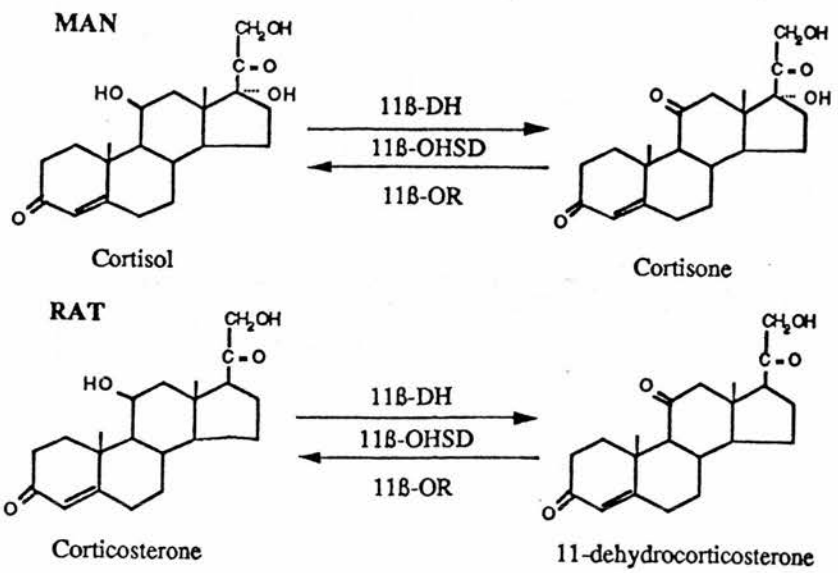



**Figure 1.7:**
Principal reactions catalysed by 11ß-hydroxysteroid dehydrogenase in man and rat. 11ß-DH, 11ß-dehydrogenase and 11ß-OR, 11ß-reductase activities of 11ß-hydroxysteroid dehydrogenase (11ß-OHSD).

Initially, the presence of 11ß-OHSD activity in liver led to the proposal that 11ß-OHSD may be a mechanism involved in the metabolism and clearance of cortisol. However, demonstrations of enzyme activity in other tissues, and the knowledge that the 11ß-reductase component of 11ß-OHSD is very active in liver (Hellman et al., 1971), suggested that this was an oversimplification of the role of 11ß-OHSD. It was not until the late 1970's, that the importance of 11ß-OHSD became apparent with the description of congenital and acquired 11ß-OHSD deficiencies in man.

### 1.3.2 11ß-OHSD Deficiency

#### 1.3.2.1 *Congenital Deficiency of 11ß-OHSD: The Syndrome of Apparent Mineralocorticoid Excess*

Congenital 11ß-OHSD deficiency is a rare abnormality, but one which can lead to severe and often fatal clinical hypertension. Indeed there have been only 21 reported cases in children, and 1 adult (Shackleton & Stewart, 1990) to date. In 1974, Werder described the first reported case of a 3 year old girl suffering from severe hypertension and hypokalaemia (Werder et al., 1974). Plasma renin activity was undetectable and mineralocorticoid excess was suspected. However plasma aldosterone was low and no other mineralocorticoids could be identified. A similar case of hypertension and hypokalaemia was reported a few years later (New et al., 1977; Ulick et al., 1977; 1979). That cortisol to cortisone metabolism was impaired in this syndrome was demonstrated by raised urinary levels of cortisol metabolites (tetrahydrocortisol [THF] and allo-THF) with respect to those of cortisone (tetrahydrocortisone [THE]) (Fig. 1.8). However again, there was apparently no increase in circulating mineralocorticoids and therefore the syndrome became known as "apparent mineralocorticoid excess" or AME.

In 1983, Oberfield et al., reported sodium retention, potassium loss and hypertension following ACTH and cortisol infusion into a patient suffering from AME (Oberfield et al., 1983). The hypertensive effects appeared to be mediated by MR since spironolactone (an 'aldosterone'-MR antagonist) led to a reduction in blood pressure, natriuresis and antikaliuresis. In addition dexamethasone treatment which binds selectively to GR reversed the hypertensive syndrome in a similar way to spironolactone. It was therefore suggested that cortisol itself may be the hypertensive agent through one of two mechanisms; (i) a defect in MR such that cortisol had an abnormally high affinity for the receptor. However this would mean that there would have to be a defect in MR as well as impaired conversion of cortisol to cortisone, which is unlikely. (ii) the normal metabolism of cortisol to cortisone may be impaired in MR containing tissues such that cortisol accumulated and then activated MR (Oberfield et al., 1983). In 1988, this hypothesis was expanded to suggest that 11ß-OHSD activity was impaired in AME such

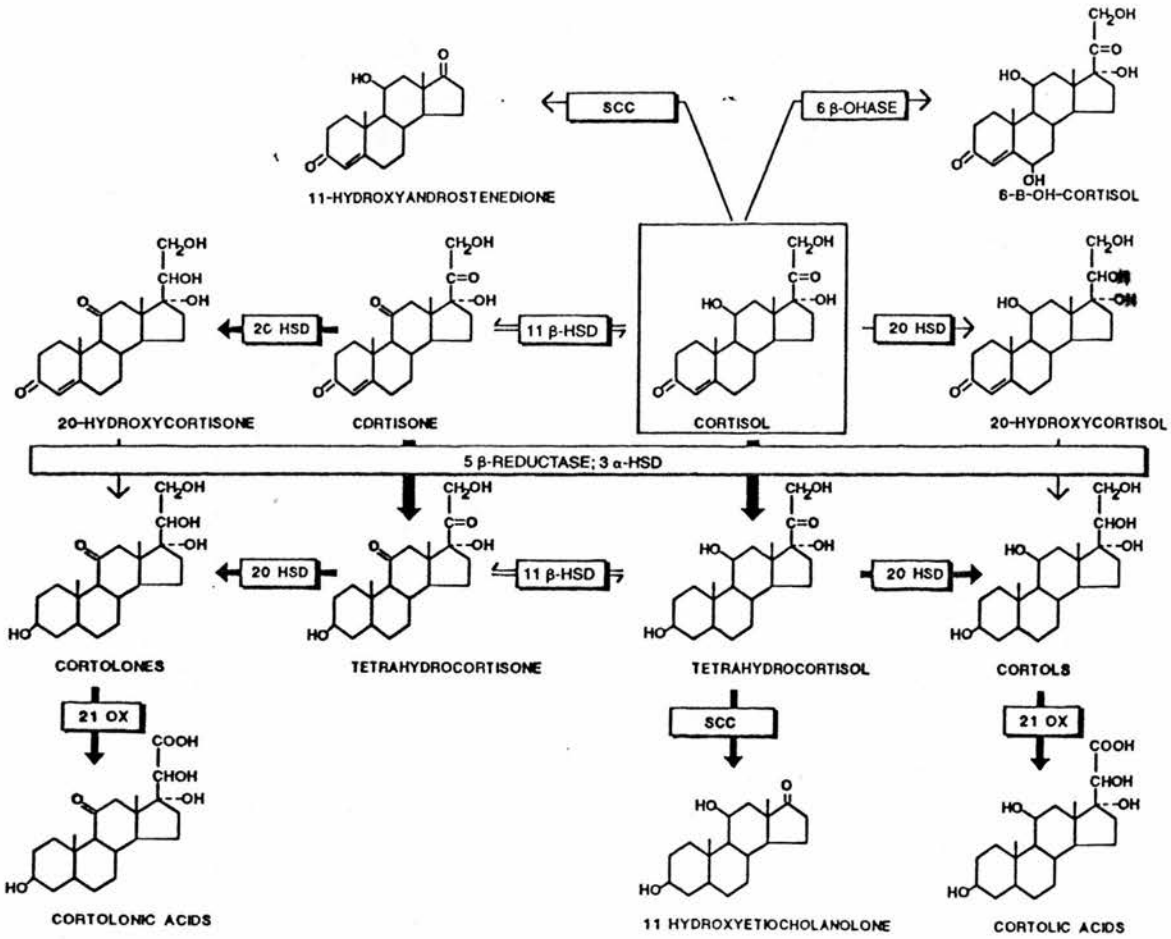



**Figure 1.8:**

Pathways involved in the metabolism of cortisol. SCC = side chain cleavage; 6ß-OHASE = 6ß-hydroxylase; 20 HSD = 20-hydroxysteroid dehydrogenase; 11ß-HSD = 11ß-hydroxysteroid dehydrogenase; 21 OX = 21 oxidase. Taken from Orth et al., 1991.

that intra-renal cortisol levels would be increased and could spill over to occupy and activate MR (Stewart et al., 1988) (Fig. 1.9).

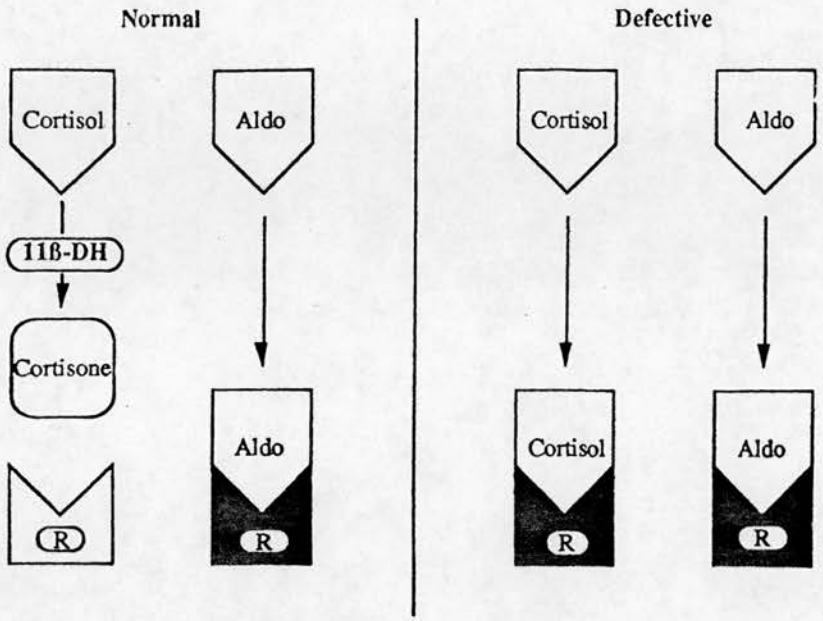



**Figure 1.9:**
11ß-hydroxysteroid dehydrogenase: enzyme mediated receptor protection. (illustration by Brian Walker).

1.3.2.2 *Acquired Deficiency of 11ß-OHSD: Liquorice-Induced Hypertension*

An important factor involved in testing the new hypothesis as to whether cortisol activates MR when 11ß-OHSD is deficient, was to come from the parallels between AME and the effects of liquorice addiction. Liquorice abuse has long been known to result in hypertension and kalliuresis which can be reversed by spironolactone treatment (Salassa et al., 1962). The effects of liquorice abuse were thought to be due to the active component of liquorice - glyccyrhetinic acid (GE) - binding to MR (Ulmann et al., 1975; Armanini et al., 1983). However since dexamethasone reversed the hypertensive symptoms of liquorice ingestion (Hoefnagels & Kloppenborg, 1983), and sodium retention was absent in patients without intact adrenal glands (Borst et al., 1953), this suggested that the presence of cortisol or another ACTH-dependent steroid was required. Therefore a direct effect of GE on MR could not be responsible for the hypertensive

effects of liquorice. However it was possible that GE may mediate its effects by inhibiting the action of 11ß-OHSD. This hypothesis was tested in volunteers taking liquorice. The resulting hypertensive symptoms were indicative of inhibition of 11ß-OHSD (Stewart et al., 1987). Parallel studies in animals confirmed that GE and glycyrrhizic acid (GI; which is converted to GE *in vivo*) inhibit renal 11ß-OHSD both *in vitro* and *in vivo* (Monder et al., 1989). Furthermore, carbenoxolone (the hemisuccinate derivative of GE) has been shown to potentiate the mineralocorticoid effect of corticosterone in intact rats (Souness & Morris, 1989) and in toad bladder mucosa (Brem et al., 1989; Gaeggeler et al., 1989). However, carbenoxolone may also inhibit 5ß-reductase (Latif et al., 1990), and 15-hydroxyprostaglandin dehydrogenase (Peskar et al., 1976), thus placing limitations on interpretation of the effects of carbenoxolone as being solely related to glucocorticoid or indeed steroid metabolism (Monder, 1991a).

1.3.2.3 *11ß-OHSD in Kidney: Tissue-Specific Protector of Renal MR*

Studies of congenital and acquired deficiency of 11ß-OHSD indicated that cortisol has the ability to bind to and activate renal MR as well as GR. How therefore can renal MR selectively bind aldosterone *in vivo*, when free circulating levels of cortisol or corticosterone are 100 to 1000-fold in excess of aldosterone? Cortisol/corticosterone binding globulin was initially proposed as the specificity conferring mechanism (Sheppard & Funder, 1987). However, MR selectivity is maintained in 10 day old rats which express relatively low levels of the binding globulins (Sheppard & Funder, 1987). An alternative suggestion that 11ß-OHSD, by catalysing the conversion of active to inactive glucocorticoids, could confer aldosterone selectivity on renal MR was originally demonstrated experimentally by two studies which both led to the same conclusions (Edwards et al., 1988; Funder et al., 1988).

The first step in both studies worked on the assumption that if 11ß-OHSD confers selective aldosterone binding to MR in mineralocorticoid tissues, then 11ß-OHSD activity should be found in these tissues, and not in other organs which contain MR. Indeed, both groups demonstrated 11ß-OHSD activity in kidney, parotid and colon, but little activity was found in heart and hippocampus. Funder et al. (1988) then went on to demonstrate that treatment of rats with carbenoxolone prior to measuring 11ß-OHSD activity *in vitro* resulted in inhibition of the conversion of cortisol to cortisone. In addition, it was surmised that if 11ß-OHSD converted corticosterone to 11-dehydrocorticosterone to protect MR from glucocorticoid exposure, then 11-dehydrocorticosterone must have low affinity for MR. Indeed it was shown in cytosolic preparations of MR and GR, that 11-dehydrocorticosterone was ineffective at competing for receptor binding with aldosterone, corticosterone and dexamethasone (Funder et al., 1988).

Localisation of renal 11ß-OHSD using a polyclonal antibody raised against purified rat liver 11ß-OHSD (Lakshmi & Monder, 1988), indicated that the enzyme was predominantly located in proximal tubules leading to the suggestion that the enzyme was a paracrine protector of renal MR which are principally located in distal tubules and collecting tubules. No immunoreactive staining was detected in hippocampal or heart tissue sections (Edwards et al., 1988).

Both groups then went on to demonstrate that corticosterone could bind to renal MR when 11ß-OHSD was inhibited by carbenoxolone. This was achieved by injecting tracer amounts of [$^3$H] aldosterone or [$^3$H] corticosterone in the presence or absence of carbenoxelone into previously adrenalectomised rats. Funder et al., then measured binding of the steroids to cytosolic receptors, while Edwards et al., localised steroid binding in kidney tissue sections exposed to autoradiographic film. Essentially however, both groups found that corticosterone exhibits no perceptible binding in kidney in an intact system. However, prior treatment with carbenoxolone or glycyrrhizic acid permits corticosterone binding in kidney, presumably to mineralocorticoid receptors, since corticosterone binding is indistinguishable from that of aldosterone (Fig. 1.10).

Clearly, congenital and acquired deficiencies of 11ß-OHSD in addition to experimental evidence have supported the proposal that the physiological role of 11ß-OHSD in kidney is to convert active to inactive glucocorticoids thus regulating glucocorticoid access to the otherwise non-selective renal mineralocorticoid receptors, and therefore preventing symptoms of apparent mineralocorticoid excess.

### 1.3.3 Further Characterisation of 'Liver-Type' 11ß-OHSD

It was originally proposed that 11ß-OHSD may consist of two separate enzymes encoding separate dehydrogenase and reductase activities. To determine if this was indeed the case, and to further characterise the enzyme, 11ß-OHSD was purified from rat liver microsomes, and found to possess only dehydrogenase activity thus supporting the 'two enzyme hypothesis'. However with the cloning of 11ß-OHSD from a rat liver cDNA library, and expression in mammalian cells, it became apparent that both dehydrogenase and reductase activities were encoded by a single protein. These studies are reviewed below.

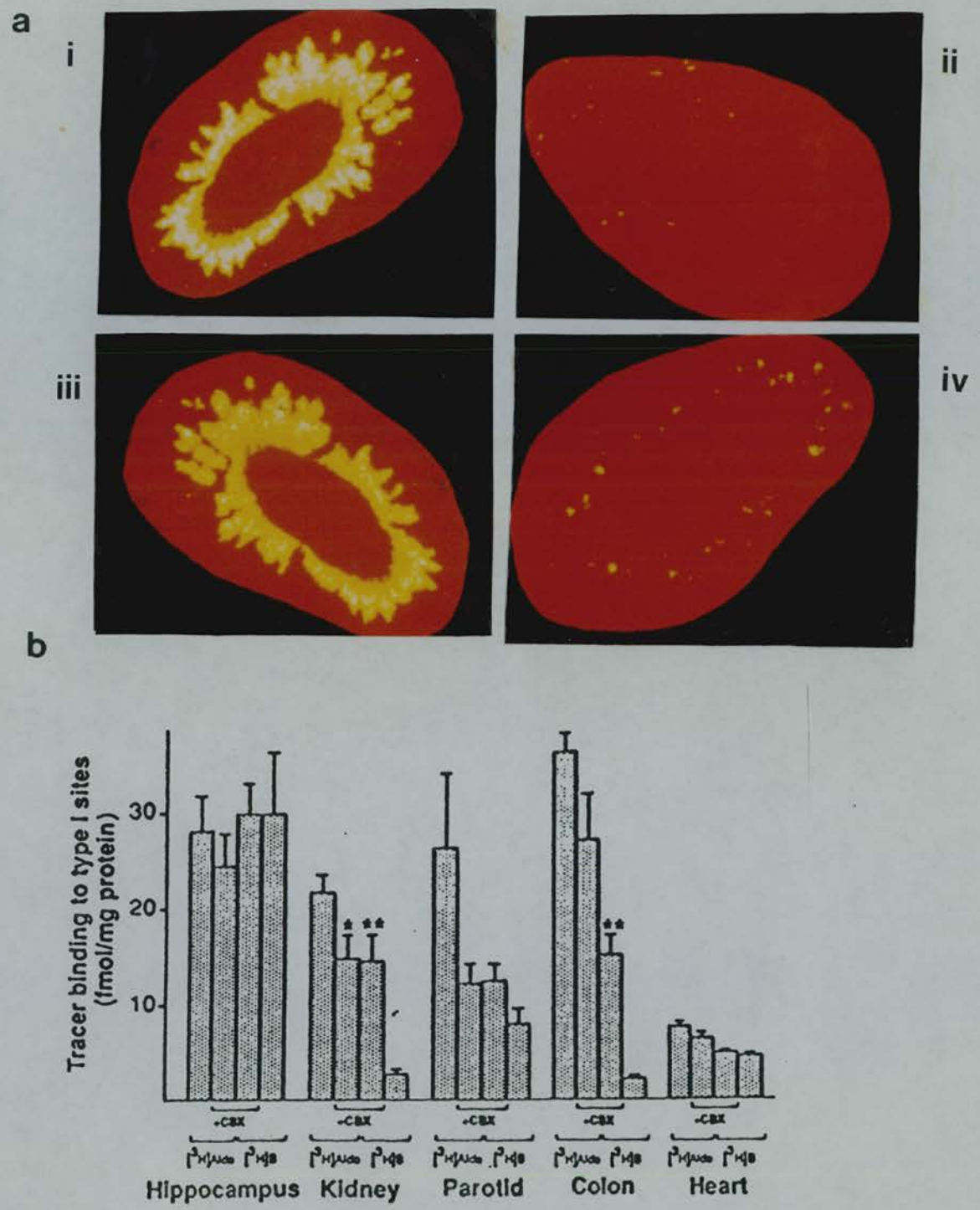



**Figure 1.10:**

*In vivo* [$^3$H] corticosterone binding to renal MR in the presence or absence of prior carbenoxolone treatment, in comparison to [$^3$H] aldosterone binding to renal MR. (a) Taken from Edwards et al., 1988; (i) [$^3$H] aldosterone binding, (ii) [$^3$H] corticosterone binding, (iii) [$^3$H] corticosterone binding following carbenoxolone treatment, (iv) [$^3$H] corticosterone binding in the presence of excess unlabelled corticosterone. (b) Taken from Funder et al., 1988; Aldo = aldosterone; B = corticosterone; CBX = carbenoxolone.

1.3.3.1 *Purification of 11ß-OHSD*

It was originally suggested that 11ß-dehydrogenase and 11ß-reductase activities exist as two separate enzymes at least in lung (Abramovitz et al., 1982) and liver (Lakshmi & Monder, 1985). In an attempt to determine if this was the case, Lakshmi & Monder (1988) purified 11ß-OHSD from rat liver microsomes. Previous attempts at purifying 11ß-OHSD were largely unsuccessful (Hurlock & Talalay, 1969; Bush et al., 1968) since the enzyme was denatured when it was removed from its membrane-bound location. However, it was found that if the native membrane environment was replaced by a synthetic detergent (Lakshmi & Monder 1985), 11ß-OHSD could be solubilised in an active state. Since rat liver 11ß-OHSD is NADP-dependent, purification of the enzyme was achieved by single step NADP-agarose affinity chromatography (Lakshmi & Monder, 1988). The purified enzyme was found to exclusively express 11ß-dehydrogenase activity in the absence of 11ß-reductase, supporting the suggestion that the two activities of 11ß-OHSD originate from separate structural entities. This was supported by the finding that AME patients are deficient in 11ß-dehydrogenase activity while 11ß-reductase activity is unaffected. Homogenous rat liver 11ß-dehydrogenase was found to be a glycoprotein with a monomer molecular weight of 34kD (Lakshmi & Monder, 1988) which exhibits a $K_m$ for cortisol of approximately 17µM and corticosterone 2µM (Monder & Lakshmi, 1989; Monder et al., 1991).

1.3.3.2 *Production of Antisera Against Purified Rat Liver 11ß-OHSD*

Three polyclonal antibodies were generated (56-125, 56-126 and 56-127) by injection of purified 11ß-OHSD into three rabbits (Monder & Lakshmi, 1990). The antisera were immunoprecipitins, but 11ß-dehydrogenase activity was not completely inhibited by the antibody suggesting that the epitopic regions did not correspond to the active site of the enzyme. Two of the antibody preparations, 56-125 and 56-126, produced tissue-specific staining patterns of 11ß-OHSD antigen by western blotting. With antiserum 56-125, a single enzyme of 34kD (corresponding to the purified 11ß-OHSD) was detected in liver, kidney, testis and lung microsomes, while an additional 40kD band was detected in kidney homogenates. Brain microsomes and homogenates showed additional cross-reaction with a 26kD band, but in contrast, anti-serum 56-126 revealed no antigen. In other tissues, antibody 56-126 showed a similar pattern of staining, although an additional 68kD band in liver and kidney was detected, which may be due to dimerisation of the 34kD species. Indeed the structurally related (Krook et al., 1990a, 1990b; section 1.3.4) bacterial 3α, 20ß-hydroxysteroid dehydrogenase has been crystallised, and shown to form multimeric complexes (Ghosh et al., 1991). However the 26kD species in brain, the 40kD species in kidney, and an additional 47kD cross-reacting

species in testis remain unexplained, but are suggestive of more than one isoform of 11ß-OHSD (Monder & Lakshmi, 1990).

In an independent study, a mouse anti-11ß-OHSD monoclonal antibody was generated (Castello et al., 1989) which detected a single antigenic species in kidney of 35kD. The mouse antibody bound both 11ß-dehydrogenase and 11ß-reductase activities as shown by binding of the antibody to solubilised kidney proteins followed by incubation of the complexes with [$^3$H] corticosterone or [$^3$H]11-dehydrocorticosterone and monitoring metabolite production by HPLC.

1.3.3.3 *Cloning of Rat 'Liver-Type' 11ß-OHSD*

A rat liver cDNA library was screened using the rabbit anti-11ß-OHSD serum (Agarwal et al., 1989). One of three clones which bound the anti-serum was subcloned into a plasmid vector for further analysis. The insert of this plasmid (p11-DH) was 1265 base pairs (bp) long, and when sequenced, revealed an 861 bp open reading frame predicting a polypeptide of 287 residues with a molecular weight of 31,774 kD. The difference between this and the predicted 34kD of the purified protein, may be due to glycosylation; there are two putative N-linked (Asn-X-Ser) glycosylation sites on the predicted sequence at residues 158-160, and 203-205.

The 11ß-OHSD cDNA was cloned into an expression vector containing the SV40 promoter and transfected into chinese hamster ovary (CHO) cells. 11ß-dehydrogenase and 11ß-reductase activities were measured by the conversion of [$^3$H]-corticosterone to [$^3$H] 11-dehydrocorticosterone and vice versa in the cell medium over 20h. In contrast to the purified native 11ß-OHSD protein, recombinant 11ß-OHSD was found to express roughly equal 11ß-dehydrogenase and 11ß-reductase activities. Expression of 11ß-OHSD in human osteosarcoma cells infected with vaccinia virus confirmed the finding of equal dehydrogenase and reductase activities with a $K_m$ of 2.14µM for corticosterone at pH 8.5 (Agarwal et al., 1990). Although cloning of rat liver 11ß-OHSD demonstrated that this isoform of the enzyme is bi-directional, while purification studies suggested the existence of separate enzymes responsible for 11ß-dehydrogenase and 11ß-reductase activities, the two hypotheses are not mutually exclusive. The 11ß-reductase component of 11ß-OHSD has been shown to be labile, and thus under some conditions the 11ß-dehydrogenase activity can be studied in the absence of the reverse activity (Lakshmi & Monder, 1985).

Recently a human 11ß-OHSD cDNA has been cloned from a human testes cDNA library using the rat liver cDNA as a probe (Tannin et al., 1991). The open reading frame of this cDNA is 876bp which predicts a protein of 292 amino acids. The sequence is 77% identical to the rat liver isoform at the amino acid level. Using the human 11ß-OHSD cDNA as a probe, the 11ß-OHSD gene was localised to chromosome 1, and was

then isolated from a chromosome 1-specific human genomic library. The human gene consists of 6 exons and is at least 9kb in length (Tannin et al., 1991). In addition, an ovine 11ß-OHSD cDNA has been isolated from a sheep liver cDNA library using the rat liver 11ß-OHSD cDNA as a probe. This 11ß-OHSD cDNA has a 72% sequence homology with the rat liver cDNA (Yang et al., 1992).

#### 1.3.3.4 *'Liver-Type' 11ß-OHSD mRNA Expression*

Northern analysis of rat liver 11ß-OHSD revealed a single, 1700 nucleotide (nt) mRNA species in liver, testis, ovary, lung, vascular tissue and various brain regions (Agarwal et al., 1989; Moisan et al., 1990a). In contrast, rat kidney expresses at least four mRNA species (Krozowski et al., 1990). In renal cortex and cortex/medulla, 1500nt, 1600nt and 1900nt species of 11ß-OHSD exist, while in renal papilla a single 1700nt species (indistinguishable from the species expressed in liver) was detected. Although deadenylation studies demonstrated that some may be due to variations in the polyadenylation chain length, other transcripts could not be attributed to this mechanism, and were proposed to represent products of a separate 11ß-OHSD gene (Krozowski et al., 1990).

The recent identification of a 5' truncated 11ß-OHSD cDNA species from the kidney, which lacks the region encoding the first 26 amino acids of the full length cDNA, and in addition has a unique 5' untranslated region appeared to support the hypothesis of a second 11ß-OHSD gene (Mercer et al., 1991; Krozowski et al., 1992). However, cloning of the rat 'liver-type' 11ß-OHSD gene promoter demonstrated that the multiple species of renal 11ß-OHSD mRNA are a result of differential promoter usage of a single gene (Moisan et al., 1992b). Using primer extension and RNase protection analyses, it was shown that the major transcription start site in liver is 105bp upstream of the start site of translation. This start site is also the predominant transcription initiation site in kidney. Two additional transcription start sites are also utilised in kidney. The truncated cDNA represents transcriptional initiation in the first intron, but this species may not be translated (discussed in section 1.3.5.1). The second species has an extended 5' untranslated region, but no in-phase open reading frame suggesting that this sequence will produce the same translated protein as the full-length protein encoded from the major transcription start site (Moisan et al., 1992b). The extended 5' untranslated region, in common with the related 17ß-hydroxysteroid dehydrogenase gene in placenta (The et al., 1989), may alter stability and translatability of the mRNA by formation of secondary structures such as stem loops within complementary regions of the RNA (Kozak, 1991).

### 1.3.4 The Short Chain Alcohol Dehydrogenase Superfamily

The deduced amino acid sequence of the rat liver, human and ovine 11ß-OHSD proteins is related to other members of a short-chain alcohol dehydrogenase gene family, which includes prokaryotic and eukaryotic enzymes involved in nitrogen fixation, the metabolism of sugars, steroids, aromatic hydrocarbons and prostaglandins as well as the synthesis of antibiotics (Baker et al., 1990a; 1990b; Krook et al., 1990; Tannin et al., 1991). The superfamily comprises enzymes which are approximately 300 amino acids in length and function independently of metal co-factors. 11ß-OHSD shares the most significant sequence similarity with the bacterial enzyme 3α, 20ß-hydroxysteroid dehydrogenase from Streptomyces hydrogenans (Marekov et al., 1990), which is involved in the reversible oxidation of 3α and 20ß hydroxyl groups of androstane and pregnane derivatives. Although human 17ß-OHSD and human 3ß-OHSD are also members of the short chain alcohol dehydrogenase family, they are not closely related to 11ß-OHSD.

Alignment of members of this family reveals 9 residues which are conserved in all proteins. These residues are therefore likely to be structurally or functionally important. 3 of these residues are in an area near the amino terminus that is similar to known nucleotide co-factor binding sites of other enzymes such as yeast alcohol dehydrogenase (Jornvall et al., 1981). The three dimensional structure of 3α, 20ß-hydroxysteroid dehydrogenase is now known (Ghosh et al., 1991), and indeed the conserved region near the amino terminus does form part of the nucleotide co-factor binding site. Two other conserved residues have been shown to lie in close proximity with the co-factor binding site, and have been proposed to participate in the catalytic functioning of the enzyme by facilitating transfer of a hydride radical from the steroid to the co-factor (Ghosh et al., 1991).

### 1.3.5 11ß-OHSD-2?

Although cloning of the rat liver 11ß-OHSD cDNA demonstrated that a single enzyme was capable of encoding both 11ß-dehydrogenase activity and 11ß-reductase activity (Agarwal et al., 1989), several lines of evidence are inconsistent with one enzyme being responsible for all of the physiological actions of 11ß-OHSD.

Use of polyclonal antibodies raised against purified rat liver 11ß-OHSD have been used to demonstrate the location of 11ß-OHSD in kidney. Several of these studies demonstrated 11ß-OHSD staining in the proximal convoluted tubules (Edwards et al., 1988; Rundle et al., 1989), whereas mineralocorticoid receptors are located in the distal convoluted tubules and cortical collecting duct cells (Rundle et al., 1989; Farman et al.,

1991). This led to the proposal that 11ß-OHSD protected renal MR from exposure to glucocorticoids by a paracrine mechanism (Edwards et al., 1988), although it has subsequently been shown that 'liver-type' 11ß-OHSD mRNA expression is present in both proximal and distal tubules (Yau et al., 1991). In addition, 11ß-OHSD activity has been demonstrated in microdissected rabbit distal tubules as well as proximal tubules (Bonvalet et al., 1990), and primary monolayer cultures of rabbit cortical collecting ducts convert corticosterone to 11-dehydrocorticosterone very effectively (Naray Fejes-Toth et al., 1991). Thus the aldosterone-sensitive regions of kidney tubules (cortical collecting tubules) express high 11ß-OHSD activity, but no apparent protein, while aldosterone-insensitive regions (proximal tubules) appear to express substantial amounts of 11ß-OHSD mRNA and protein.

Kinetic studies on the liver-derived 11ß-OHSD indicated that the Km of 11ß-OHSD for corticosterone was approximately 2μM, and that for cortisol was 17μM (Agarwal et al., 1990). However, physiological glucocorticoids circulate 'free' at low nM concentrations, and MR exhibit low nM (~0.5nM) affinity for glucocorticoids (Arriza et al., 1987; Krozowski & Funder, 1983). Thus it seems unlikely that the 'liver-type' 11ß-OHSD would be an effective mechanism for conferring aldosterone selectivity on renal MR.

Ontogenic studies of 11ß-OHSD activity (Moisan et al., 1992a) and mRNA expression (Krozowski et al., 1990; Moisan et al., 1992a) have shown that although 11ß-OHSD activity in kidney is high at postnatal day 1, expression of the 'liver-type' 11ß-OHSD mRNA in kidney is low at postnatal day 1 and increases until reaching maximum levels after postnatal day 15 (Moisan et al., 1992a). This in conjunction with studies presented in chapter 5, suggest that in the face of low 'liver-type' 11ß-OHSD mRNA expression, another isoform of 11ß-OHSD may be responsible for the high enzyme activity observed in kidney during development.

Finally, patients suffering from apparent mineralocorticoid excess appear to exhibit a selective deficiency in 11ß-dehydrogenase activity, while conversion of inactive to active glucocorticoids remains unaffected. Indeed separate and distinct symptoms are presented in patients suffering with an apparently isolated defect in 11ß-reductase activity (Taylor et al., 1984; Philipou & Higgins, 1985). In addition, recent genetic analysis of 4 patients suffering from AME and the parents of one of these patients have indicated that there are no gross deletions or rearrangements of the known 11ß-OHSD gene (Nikkila et al., 1993). Interestingly, there was also no obvious abnormalities of the gene in one patient suffering from clinical reductase deficiency.

1.3.5.1 *11ß-OHSD-1B*

Evidence for the existence of an alternative isoform of 11ß-OHSD was presented in 1989, when it was demonstrated that a higher affinity 11ß-OHSD species was present in rat liver microsomes (Monder & Lakshmi, 1989). However this species only accounted for approximately 10% of total 11ß-OHSD activity, and has not been demonstrated in the purified enzyme.

It has also recently been speculated that the truncated 11ß-OHSD species found in kidney (11ß-OHSD 1B) may be an important isoform of 11ß-OHSD. Indeed, it has been shown that 11ß-OHSD 1B mRNA is only expressed in kidney, predominantly in renal medulla (Mercer et al., 1991; Krozowski et al., 1992). In addition, 11ß-OHSD 1B when transfected into COS cells, is recognised by the polyclonal antibody 56-125. Unlike the full length 11ß-OHSD, the truncated species has an apparent molecular weight of 26kD (the same size as the species expressed in brain). In contrast, kidney homogenates do not express a 26kD 11ß-OHSD species despite an abundance of 11ß-OHSD 1B mRNA, suggesting that under normal physiological situations, 11ß-OHSD 1B may not be translated. Furthermore, recombinant expressed 11ß-OHSD 1B does not metabolise [$^3$H] corticosterone and is therefore inactive (Krozowski et al., 1992, Obeid et al., 1993) at least in the cell lines used. In addition this species of 11ß-OHSD is not expressed in other aldosterone-sensitive tissues such as colon and parotid and is therefore unlikely to be a suitable protector of MR in mineralocorticoid-responsive tissues. The role of 11ß-OHSD 1B is as yet unknown, but when aligned with other members of the short chain alcohol dehydrogenase family, it was revealed that the truncated 11ß-OHSD isoform still contains the conserved residues which have been proposed to form the co-factor binding site (Krozowski, 1992), suggesting that if translated, 11ß-OHSD 1B may have some functional significance. Since the first 26 hydrophobic residues are missing from 11ß-OHSD 1B, it would be likely to have a different cellular location to the full length 11ß-OHSD, and may have a role as a binding protein.

Several reports now exist which support that the 11ß-OHSD activity in mineralocorticoid-target tissues is a distinctive enzyme with different co-factor specificity, different molecular weight and different substrate affinities. This evidence is reviewed below.

1.3.5.2 *NAD-dependent isoform of 11ß-OHSD*

Recently a novel activity staining technique has indicated the existence of an NAD-dependent isoform of 11ß-OHSD (Mercer & Krozowski, 1992). Generally, adenine dinucleotide dependent enzymes favour either the use of NAD or NADP, but rarely express no preference for one or the other. The NAD-dependent activity of 11ß-OHSD

was demonstrated in rat kidney distal tubules by looking at the production of diformazan from tetrazolium blue using 11ß-hydroxyandrostenedione as substrate in the presence of NAD Mercer & Krozowski, 1992). NADP was not a functional co-factor in this assay. NAD-dependence of 11ß-OHSD using this assay has also been directly demonstrated in pig kidney using cortisol as a substrate (Provencher et al., 1992). Localisation of the NAD-dependent isoform of 11ß-OHSD is consistent with localisation of mineralocorticoid receptors (Rundle et al., 1989; Farman et al., 1991). Similarly, another study looking at the tissue-specific distribution of NAD-dependent 11ß-OHSD activity indicated that this isoform is more closely associated with aldosterone-selective mineralocorticoid tissues such as colon and kidney. NAD-dependent 11ß-OHSD activity was also demonstrated in placenta, while other tissues such as liver, testes, hippocampus and the vasculature exhibit little NAD-dependent 11ß-OHSD activity (Walker et al., 1992).

The placenta also expresses high levels of 11ß-OHSD activity which is thought to protect the foetus from the deleterious effects of high maternal glucocorticoids (Lopez-Bernal et al., 1980; Murphy et al., 1974). Physicochemical and kinetic studies on partially purified human placental 11ß-OHSD suggest that it is likely to be a different isoform from rat liver 11ß-OHSD (Brown et al., 1993a). For example, the Km of human placental 11ß-OHSD for corticosterone is 14nM and for cortisol is ~55nM, both of which are 100-fold lower than 'liver-type' 11ß-OHSD. In addition, human placental 11ß-OHSD is NAD-dependent, does not appear to labile and has different detergent solubility and pH optimum than rat 'liver-type' 11ß-OHSD (Brown et al., 1993a). These differences are not a reflection of species specificity, since rat placental 11ß-OHSD is also NAD-dependent and has very similar characteristics to the human isoform. Over 1000-fold purification of human placental 11ß-OHSD was achieved by AMP-affinity chromatography using NAD as the protein eluter. SDS-PAGE of the purified fractions indicate a protein of Mr 40kD which consistently segregates with the NAD-dependent 11ß-OHSD activity. Although a 40kD band was identified in kidney by western blotting using polyclonal antisera to the purified rat liver 11ß-OHSD, human placental 11ß-OHSD did not cross-react with these antisera (Brown et al., 1993a), nor did rat kidney 11ß-OHSD which had been passed over the column (Brown et al., 1993b). Similarly, it will be demonstrated in chapter 4 that an additional isoform of 11ß-OHSD found in kidney does not cross-react with the 'liver-type' 11ß-OHSD cDNA, suggesting that the two (or more) isoforms of 11ß-OHSD are substantially different from each other. This may be analogous to the substantial differences between the two isoforms of 5α-reductase, which exhibit only ~50% homology despite their similar functions (Andersson et al., 1991).

In light of the aforementioned studies demonstrating NAD-dependent 11ß-OHSD activity in kidney (Mercer & Krozowski, 1992; Walker et al., 1992), it may be speculated

that the high affinity placental 11ß-OHSD isoform would be a likely candidate for protecting renal mineralocorticoid receptors from glucocorticoid exposure. Indeed, it has been demonstrated recently that the NAD-dependent 11ß-OHSD activity in microsomes of renal collecting tubules has low nM Km for corticosterone and cortisol, and expresses very little reductase activity, similarly to placenta (Rusvai et al., 1993). However definitive information as to whether the NAD-dependent isoform(s) of 11ß-OHSD are the same in placenta and renal collecting ducts will require complete purification of the protein(s) and the cDNA(s) and the gene(s) which encode them.

### 1.3.6 Other Roles for 11ß-OHSD

Although the physiological role of 11ß-OHSD in kidney has been established as a protective mechanism regulating glucocorticoid access to MR in distal convoluted tubules and cortical collecting tubules, 11ß-OHSD activity and 'liver-type' mRNA expression have now been demonstrated in a number of other tissues. Thus as well as being expressed in typical aldosterone-selective mineralocorticoid target tissues, 11ß-OHSD has also been demonstrated in tissues containing predominantly GR such as liver, colon, skin and cerebellum (Fuller & Verity, 1990; Teelucksingh et al., 1990; Moisan et al., 1990b; Whorwood et al., 1992), and in tissues such as hippocampus where MR are present, but respond to glucocorticoids (Moisan et al., 1990a; Sakai et al., 1990; Lakshmi et al., 1991). The physiological role of 11ß-OHSD in these tissues is not clearly understood.

#### 1.3.6.1 *Placenta*

Epidemiological studies have implicated environmental factors acting during intrauterine growth which may increase the risk of certain clinical disorders later in life. For example, low birthweight is strongly correlated with the risk of ischaemic heart disease mortality (Barker et al., 1989a) and high blood pressure at all ages (Barker et al., 1989b; Whincup et al., 1989; Barker et al., 1990). The strongest predictor of adult hypertension is a combination of low birthweight with a large placenta (Barker et al., 1990); independent of other risk factors for hypertension such as obesity and smoking. These features have been attributed to maternal malnutrition, but they have also been found in rats with streptozotocin-induced diabetes mellitus (Robinson et al., 1988; Canavan & Goldspink, 1988) which have high maternal glucocorticoid levels (Heller et al., 1988). In addition, exogenous glucocorticoids retard foetal growth in humans and animals (Reinisch et al., 1978). Placental 11ß-OHSD protects the foetus from exposure to maternal glucocorticoids (Beitins et al., 1973) by converting active cortisol to inactive cortisone with negligible reduction of cortisone to cortisol. Foetal cortisol concentrations

are therefore much lower than maternal levels (Edwards et al., 1993). It has recently been shown that placental 11ß-OHSD activity positively correlates with birthweight and negatively with placental weight (Benediktsson et al., 1993). Thus, small foetuses with a large placentae (which have a higher risk of developing adult hypertension) have the lowest 11ß-OHSD activity, and presumably the highest exposure to glucocorticoids. Placental 11ß-OHSD has therefore been proposed as a crucial determinant of foetal exposure to maternal glucocorticoids, and in turn a determinant of later hypertension and ischaemic heart disease (Barkeret al., 1993; Edwards et al., 1993), though clearly this may only be one of several mechanisms acting to regulate intrauterine events.

1.3.6.2 *Liver*

Very little is known about the functions of 11ß-OHSD in liver. 11ß-OHSD activity is higher in liver than any other tissue, and liver was the source of the 'originally purified 11ß-OHSD. In contrast to kidney, where 11ß-OHSD converts active to inactive glucocorticoids, high levels of cortisol with respect to cortisone in the effluent of perfused cat liver indicate that in cats, equilibrium in the liver favours the active steroid, and therefore 11ß-reductase predominates (Bush et al., 1969). In addition, recent experiments suggest that 11ß-reductase activity predominates in human liver. Following oral administration of cortisone, peripheral plasma levels of cortisol, but not cortisone, rise (Stewart et al., 1990), thus indicating that substantial cortisone is activated by 11ß-reductase on first-pass through the liver. High levels of 11ß-OHSD activity in liver in conjunction with the enzyme being predominantly 11ß-reductase suggest a very different physiological role from that in kidney. However, considerable work is required to determine the physiological actions of 11ß-OHSD in liver especially since in rats at least, 11ß-OHSD activity in female liver is approximately 2-fold lower than that observed in male rat liver (discussed in Chapter 4).

13.6.3 *Testis*

11ß-OHSD plays no direct role in the metabolism of testicular steroids since none are 11-hydroxylated. However, corticosteroids are known to directly suppress testosterone secretion in rats (Saez et al., 1977) through GR-mediated actions (Bambino & Hseuh, 1981; Welsh et al., 1982). Testosterone levels are highest in foetal rats, fall at birth, and rise again during the 4th and 5th weeks postnatally. Phillips et al. (1989) showed that 'liver-type' 11ß-OHSD immunoreactivity is not apparent in foetal testes or in the testes of 5-25 day old animals. However from age 31-33 days, intense 11ß-OHSD immunoreactivity is detected in leydig cells in the testes. It has therefore been hypothesised that 11ß-OHSD activity in pubertal and adult testes inactivates corticosterone so that testosterone secretion is no longer inhibited, and sexual maturation

can proceed (Phillips et al., 1989). This theory is supported by the finding that carbenoxolone treatment of leydig cell suspensions results in potentiation of corticosterone inhibition of testosterone release (Monder, 1991b).

1.3.6.4 *Ovary*

11ß-OHSD activity, 'liver-type' mRNA and immunoreactivity are found in the ovary (Benediktsson et al., 1992) located in the oocyte and luteal bodies. 11ß-OHSD activity and mRNA have also been found in human ovaries (Murphy, 1981; Tannin et al., 1991). GR are also present in the ovary (Schreiber et al., 1982), indicating a possible role of 11ß-OHSD in modulating the glucocorticoid effects on ovarian function. Indeed it has been recently shown that of 64 patients undergoing in vitro fertilisation and embryo trnasfer, 32 had detectable 11ß-OHSD activity in cultures of their granulosa-lutein cells, and none of these 32 patients became pregnant although fertilisation was achieved in each case (Michael et al., 1993). In contrast, 76% of the remaining '11ß-OHSD-negative' patients achieved pregnancy. Thus low ovarian 11ß-OHSD activity and/or high intrafollicular cortisol may be necessary for a viable pregnancy to occur.

1.3.6.5 *Mammary Gland*

Glucocorticoids induce milk production from the mammary glands by a GR-mediated process (Jahn et al., 1987; Quirk et al., 1988). High levels of 11ß-OHSD activity have been demonstrated in the adipose tissue of the mammary gland, but activity has been shown to decrease following parturition (Quirk et al., 1990a). In addition 11ß-OHSD activity is three-fold higher in the adipose cells from pregnant rats than cells from lactating rats (Quirk et al., 1990b). Therefore it has been suggested that high 11ß-OHSD activity during pregnancy may play a role in prevention of premature milk production in response to glucocorticoids.

1.3.6.6 *Skin*

Cutaneous vasoconstriction by glucocorticoids is known to be a GR-mediated process (Marks et al., 1982), suggesting a direct effect of corticosteroids in skin. The effectiveness of topical corticosteroids on suppression of the inflammatory response is partly determined by its persistence in the area of application. 11ß-OHSD activity in the oxidative direction would therefore attenuate the effectiveness of topical hydrocortisone, and inhibition of 11ß-OHSD should potentiate the local action of glucocorticoids. To test this theory, a skin vasoconstrictor assay (which requires topical application of steroids to the forearm under an occlusive dressing) was used to show that hydrocortisone alone has minimal vasoconstrictive effects (Teelucksingh et al., 1990). However in the presence of GE, there was a marked potentiation of the vasoconstrictor response. In addition

immunohistochemistry and *in vitro* 11ß-OHSD enzyme activity assays have demonstrated the presence of 11ß-OHSD in the epidermal layers of human and nude mouse skin (Teelucksingh et al., 1990). Although this study has revealed a potential therapeutic use of 11ß-OHSD inhibition in maximising the effects of topical hydrocortisone, the physiological role of 11ß-OHSD in skin remains unclear. This is due in part to a conflicting study which reported that 11ß-reductase activity exceeds 11ß-dehydrogenase activity in cultured human skin fibroblasts (Hammami & Siiteri, 1991), and therefore that the anti-inflammatory effects of topical application of GE on human skin cannot be fully explained by the inhibition of 11ß-OHSD.

1.3.6.7 *Vasculature*

Corticosteroids are believed to play important roles in blood pressure regulation by as yet unknown mechanisms. However the presence of both MR and GR in vascular smooth muscle cells (Meyer & Nichols, 1982; Kornel et al., 1982) and heart (Funder et al., 1973; Arriza et al., 1987) indicates the possibility of a direct action of corticosteroids on these tissues. High levels of glucocorticoids could, by binding to both MR and GR, contribute to the pathogenesis of essential hypertension by stimulating vasoconstriction. MR in the mesenteric vascular arcade is aldosterone specific *in vivo* (Funder et al., 1989), leading to the proposal that 11ß-OHSD may mediate aldosterone selectivity to MR in a similar way to kidney. Glucocorticoids also have independent effects on vascular smooth muscle cells in culture which are attenuated by RU38486 indicating glucocorticoid dependence (Kornel, 1988; Nichols et al., 1985). 11ß-OHSD activity has also been reported in the vasculature (Kornel et al., 1982; Funder et al., 1989; Walker et al., 1991). 11ß-OHSD is therefore appropriately located to modulate access of glucocorticoids to vascular receptors, and could thereby influence vascular tone and blood pressure.

1.3.6.8 *Colon*

Corticosteroids increase colonic sodium absorption, probably in a manner similar to the renal distal tubules (Bastl, 1987; 1988; Halvey et al., 1988). In the distal colon, Na.K ATPase is the major sodium reabsorption mechanism (Marver, 1984). Induction of expression of rat distal colonic Na.K ATPase $\alpha 1$ and ß subunits has been recently shown to be regulated by acute dexamethasone but not aldosterone administration suggesting a GR-mediated effect (Fuller & Verity, 1990). Carbenoxolone treatment also induced Na.K ATPase subunit expression in intact but not adrenalectomised rats, suggesting that inhibition of 11ß-OHSD by carbenoxolone increases glucocorticoid occupancy of GR. In addition, GR and 11ß-OHSD mRNAs, and 11ß-OHSD activity have recently been demonstrated in rat colon (Whorwood et al., 1992).

1.3.6.9 *Brain*

As with other tissues, MR and GR mediate central corticosteroid effects. However unlike kidney, MR displays equal preference for corticosterone and aldosterone in rat brain. This suggests therefore in the presence of a 100- to 1000-fold circulating excess of corticosterone, that central MR will be almost permanently occupied by corticosterone, and thus insensitive to aldosterone. Based on these considerations, deKloet & Reul (1987) proposed a "tonic" hypothesis of steroid action in the brain. They showed that under physiological conditions, cerebral MR would be 80-90% occupied by corticosterone, thus providing a basal expression (tonic influence) of brain function for monitoring the external environment. Occupancy of GR is low at basal levels of circulating corticosterone during the diurnal trough, but increases under stress, and to a lesser extent during the diurnal peak, leading to occupancy of GR and generation of a negative feedback mechanism. Thus there are reciprocal tonic-activating actions, and feedback damping mechanisms. These mechanisms are viewed as continuous dependent on the selective occupancy of MR and GR by corticosterone. Arriza et al. (1988) have proposed that MR and GR act as a binary response system for corticosterone by both receptors activating an overlapping set of genes, the size of the response being dependent on circulating glucocorticoid levels. Van Den Berg et al. (1990) have suggested that central MR and GR mediate opposing effects of glucocorticoids and mineralocorticoids on blood pressure, consistent with other evidence that MR and GR in the brain mediate reciprocal neurochemical, neuroendocrine and behavioural responses.

Nevertheless, the brain responds to aldosterone in specific ways which cannot be mimicked by corticosterone. For example intracerebroventricular (icv) administration of aldosterone leads to elevated blood pressure which cannot be produced either by corticosterone or systemic steroid administration (Gomez-Sanchez, 1991). The hypertension induced by icv aldosterone administration is dependent on corticosterone, since adrenalectomy prevented the effect, and it was restored by exogenous corticosterone. In addition, aldosterone implants placed in the paraventricular nucleus of adrenalectomised rats has been shown to induce fat ingestion, while corticosterone stimulated carbohydrate intake (Tempel & Liebowitz, 1989).

Thus the brain presents an unusual paradox: in some brain regions responses are selectively evoked by aldosterone, whereas in others MR mediates corticosterone selective effects. The regional selectivity of brain corticosteroid receptors for mineralocorticoids and glucocorticoids has not yet been determined. For several years, CBG was accepted as the specificity conferring mechanism, but in the same way as for kidney, this proposal was withdrawn since selectivity was not altered in animals with relatively little CBG (Funder, 1986; de Kloet & Reul, 1987). 11ß-OHSD was then proposed as a local selectivity mechanism by inactivating glucocorticoids to allow

unimpeded access of aldosterone to MR. However, original studies did not detect any 11ß-OHSD activity in whole brain homogenates (Edwards et al., 1988; Funder et al., 1988). This may be due to low co-factor levels in brain tissue (Glock & McLean, 1955), since addition of NADP to *in vitro* enzyme assays significantly increased the conversion of corticosterone to 11-dehydrocorticosterone by 11ß-OHSD in various regions of the brain, with highest activity in hippocampus, cerebellum and cortex (Moisan et al., 1990a; 1990b; Lakshmi et al., 1991). Other regions have low but significant levels of 11ß-OHSD. This was substantiated by immunohistochemical localisation of brain regions with antibodies raised against purified 11ß-OHSD (Lakshmi et al., 1991), and by *in situ* hybridisation using cRNA probes (Moisan et al., 1990a; 1990b) corresponding to the 'liver-type' 11ß-OHSD cDNA (Agarwal et al., 1989).

Immunolocalisation of 11ß-OHSD in the hippocampus in the CA1-4 regions and dentate gyrus coincided with the distribution of MR (Sakai et al., 1992) supporting the view that 11ß-OHSD may regulate glucocorticoid access to corticosteroid receptors in hippocampus. In addition, utilisation of the quantitative 2-deoxyglucose uptake method in conscious rats, provided evidence of the *in vivo* relevance of 11ß-OHSD in brain (Seckl et al., 1991). It was shown that *in vivo* GE administration increases glucose utilisation in hippocampus, particularly the CA3 field, and also several regions of the hypothalamus (Seckl et al, 1991). In the hippocampus, as in other brain regions, glucocorticoids and mineralocorticoids must both be present in some crucial relationship for optimal function to occur. Neurones containing MR and GR must be able to manipulate glucocorticoid and mineralocorticoid levels to allow activation of available receptors. This may require that corticosterone concentrations are adjusted in such a way as to maximise MR and/or GR occupation in line with the needs of the cell at a particular time. Alternatively, glucocorticoid metabolism may proceed extensively to allow aldosterone binding to MR. How 11ß-OHSD activity is regulated to permit these functions is unknown.

11ß-OHSD is unlikely to simply provide selective access of aldosterone to MR in brain. In some brain regions for example, 11ß-OHSD may regulate glucocorticoid access to GR. Cerebellum contains no measurable MR but has well defined GR (Sousa et al., 1989). In addition, 11ß-OHSD activity and mRNA expression are high in cerebellum (Moisan et al., 1990b). Therefore 11ß-OHSD may also regulate access of glucocorticoids to GR.

### 1.3.7 Regulation of 11ß-OHSD

The majority of the studies reviewed below describing the regulation of 11ß-OHSD by corticosteroids, sex steroids and thyroid hormone have been carried out by measuring

changes in 11ß-OHSD activity. However, measurements of enzyme activity alone do not necessarily distinguish between the possible isoforms of 11ß-OHSD. Thus opposite effects in different tissues could be due to effects of the steroids or hormones on different 11ß-OHSD isoforms. This is further discussed in Chapter 5.

1.3.7.1 *Corticosteroids*

It is entirely conceivable that glucocorticoids may modulate their own metabolism by regulating 11ß-OHSD activity. Indirect evidence supporting this concept comes from the demonstration that stress in the form of cold exposure (2°C ambient temperature for 7 days) or starvation (withdrawal of food pellets for 3 days) leads to an increase in the reductase component of 11ß-OHSD in rat lung (Nicholas & Lugg, 1982). In addition, adrenalectomy results in attenuation of cortisone to cortisol conversion (Nicholas & Lugg, 1982). Human foreskin fibroblast cell 11ß-OHSD activity in both oxidative and reductive directions is increased 3-fold following incubation with 100nM dexamethasone (Hammami & Siiteri, 1991). This effect is blocked by the glucocorticoid receptor antagonists RU38486 and dexamethasone mesylate and by cycloheximide and actinomycin D suggesting that protein synthesis and mRNA synthesis are involved in the up-regulation of 11ß-OHSD activity. However, glucocorticoid regulation of 11ß-OHSD appears to be tissue-specific since 11ß-OHSD activity in kidney does not appear to be affected by adrenalectomy or dexamethasone treatment (Moisan et al., 1990c; Smith & Funder, 1991), while hippocampal 11ß-OHSD activity is increased by dexamethasone treatment and decreased by adrenalectomy compared to sham-operated controls (Moisan et al., 1990c).

Glucocorticoids increase lung maturation in late gestation by increasing the amount of pulmonary surfactant on the alveolar surface (Kotas & Avery, 1971). It is interesting to note that in human foetal lung for example, 11ß-OHSD activity in early pregnancy is almost entirely in the dehydrogenase direction (ie inactivation of glucocorticoids), while premature births appear to have little 11ß-OHSD activity in either direction, and in early childhood 11ß-OHSD in lung shows slight conversion of cortisone to cortisol (Murphy, 1978). The alteration from inactivation of cortisol to slight cortisol production as a function of gestational age has been proposed to be regulated by pituitary factors, since foetal lung from anencephalics, although showing the same overall changes, did so to a lesser extent. Furthermore, isolated late gestation perfused foetal rabbit lung has been shown to produce active cortisol in the near absence of the reverse reaction (Torday et al., 1976), and the conversion of cortisone to cortisol has been shown to increase with gestational age, although this is not affected by direct administration of dexamethasone to the foetus (Lugg & Nicholas, 1978). However in direct contradiction, Smith et al. (1973) and Pasqualini et al. (1970), both demonstrated an net conversion of cortisol to cortisone

in human foetal lung cells, as with all other foetal tissues apart from liver and intestine (Murphy, 1981). These results may not be mutually exclusive, since it has been demonstrated that human foetal lung cells diverge during growth in cell culture into populations of epithelial cells and fibroblast-like cells which can be separately cultured. The epithelial cells preferentially oxidise cortisol to cortisone, while the fibroblast-like cells favour the reverse reaction (Abramowitz et al., 1982). Therefore it has been suggested that alterations in the ratio of cell types in intact lung at particular stages of development may influence the equilibrium of 11ß-OHSD activity during gestation, although this remains to be tested.

1.3.7.2 *Androgens and Oestrogens*

Sex differences in 11ß-OHSD activity in males and females appear to develop after puberty in normal rats (Ghraf et al., 1975a). Sexually dimorphic expression of 11ß-OHSD activity is apparent in several tissues including liver (Ghraf et al., 1975a; Lax et al., 1978; 1979) and kidney (Ghraf et al., 1975a; 1975b; Smith & Funder 1991). Consistent with the observation of sexual dimorphism is strong evidence that 11ß-OHSD activity is affected by the administration and withdrawal of sex steroids. Thus, 11ß-OHSD activity in rat liver typically exhibits sexual dimorphism, with higher levels of activity found in male than female liver (Lax et al., 1978; 1979; Ghraf et al., 1975a) due to oestradiol repression of hepatic 11ß-OHSD activity in females (Lax et al., 1978). Similarly, several studies have demonstrated sexual dimorphism of renal 11ß-OHSD activity, again with higher levels found in male kidney (Ghraf et al., 1975a; 1975b; Smith & Funder, 1991). However, the role of sex steroids in the maintenance of this sex difference is unclear. Ghraf et al (1975b) showed that ovariectomy of female rats increased 11ß-OHSD activity towards normal male levels, while oestradiol treatment of intact males led to feminisation of renal 11ß-OHSD activity. In addition, gonadectomy of females had no effect on renal 11ß-OHSD activity suggesting that oestradiol represses 11ß-OHSD activity in normal female kidney (Ghraf et al., 1975b). In contrast, Smith & Funder (1991) demonstrated that oestradiol treatment of intact males led to an increase in renal 11ß-OHSD activity, while gonadectomy and/or testosterone treatment of females had no effect (Smith & Funder, 1991).

11ß-OHSD in neonatal rat testis also responds to oestrogen administration. Oestradiol decreased 11ß-OHSD activity, while in contrast, testosterone treatment had no effect (Ghraf et al., 1975c). It would therefore seem likely that 11ß-OHSD activity may also be regulated in other tissues which are responsive to sex steroids. Indeed, raised oestrogen levels either naturally towards the end of pregnancy, or in response to androstenedione administration led to increased 11ß-OHSD activity in the oxidative direction in baboon placenta (Baggia et al., 1990). This effect was reversed by

foetectomy (Baggia et al., 1990) or administration of the anti-oestrogen MER-25 (Pepe & Albrecht, 1987).

Finally, in perfused male rat lung, the reductase component of 11ß-OHSD (measured by conversion of cortisone to cortisol) is decreased following castration (Nicholas & Lugg, 1982). Thus, it appears that sex steroids affect 11ß-OHSD activity in many tissues, and indeed in a tissue-specific manner. However whether this is a direct effect of sex steroids is not clear. In rats, hypophysectomy of females leads to an abolition of sexual dimorphism, and the effects of sex steroids on hepatic 11ß-OHSD activity (Lax et al., 1978). This suggests the importance of one or more pituitary factors in the maintenance of sexual dimorphism, but the effects of pituitary hormones on 11ß-OHSD have not been studied in any detail as yet.

1.3.7.3 *Thyroid Hormone*

Regulation of 11ß-OHSD activity by thyroid hormone in addition to being tissue-specific, also appears to be species-specific. Raised thyroid hormone levels either in spontaneous hyperthyroidism, or following exogenous $T_3$ administration have been shown to increase 11ß-OHSD activity (estimated by measuring THF/THE ratios in urine) in humans (Hellman et al., 1961; Gordon & Southren, 1977). Increased conversion of cortisol to cortisone was also shown in an independent study by measuring the production of $^3H_20$ following administration of 11$\alpha$-[$^3$H]-cortisol (Zumoff et al., 1983). In contrast, in rats $T_3$ administration has been reported to decrease 11ß-OHSD activity in liver (Koerner & Hellman, 1964; Lax et al., 1979) that is apparent after only 4d continuous treatment. Hypothyroidism or thyroidectomy reverses these responses, leading to a reduction in 11ß-OHSD activity in humans (Hellman et al., 1961; Gordon & Southren, 1977; Zumoff et al., 1983), and an increase in hepatic 11ß-OHSD activity in rats (Koerner & Hellman, 1964). In addition to the species specificity, Lax et al. (1979) showed that hepatic 11ß-OHSD activity responded in opposite ways depending on the sex of the rat, such that 11ß-OHSD activity was increased by thyroidectomy in female rat, but in contrast was decreased in male rat liver.

The effects of thyroid hormone are tissue-specific. 11ß-OHSD activity in rat kidney is unaffected by thyroidectomy or $T_3$ treatment for up to 17 days (Koerner & Hellman, 1964; Lax et al., 1979; Smith & Funder, 1991). In addition, thyroid hormones do not affect 11ß-OHSD activity in the reticuloendothelial system (Dougherty et al., 1960). It has been proposed that thyroid hormones may act by controlling the levels of pyridine nucleotides (Dougherty et al., 1960). However in the presence of added co-factor, the same regulatory patterns are maintained (Koerner & Hellman, 1964). Whether thyroid hormones have a direct or indirect mechanism of action is not known.

1.3.7.4 *Other Hormones*

In contrast to the increase in placental 11ß-OHSD activity by oestrogen (Baggia et al., 1990), it has been shown that 11ß-OHSD is attenuated by pregnenolone and progesterone when incubated with placental extract *in vitro* (Lopez Bernal et al., 1980; Murphy, 1981). In addition, the cortisol and cortisone content of amniotic fluid from diabetic and non-diabetic pregnant women is identical, suggesting that insulin and glucagon do not influence 11ß-OHSD activity, at least in placenta (Baird & Bush, 1960). However in contrast, insulin has been shown to inhibit both dehydrogenase and reductase activities of 11ß-OHSD in cultured human genital skin fibroblasts (Hamammi & Siiteri, 1991).

## 1.4 Growth Hormone Secretion in Rats

It has previously been shown that hypophysectomy of female rats abolishes sexually dimorphic expression of 11ß-OHSD and in addition abolishes the effects of sex steroids on 11ß-OHSD activity (Lax et al., 1978). This suggests the importance of one or more pituitary factors in the maintenance of sexual dimorphism. If pituitary hormones regulate 11ß-OHSD, then growth hormone (GH) is likely to be a good candidate as it has been shown to mediate the sex differences of other enzymes in the liver (Gustafsson et al., 1983) and is secreted in sex-specific patterns (Fig. 1.11).

Growth hormone (GH) secretion from the anterior pituitary is regulated by at least two opposing hypothalamic peptides, somatostatin (SS) (Brazeau et al., 1973) which inhibits GH release, and GH-releasing factor (GRF) (Guillemin et al., 1982; Rivier et al., 1982) which promotes GH release. In addition, numerous other peripheral factors including nutrients, thyroid hormones, and gonadal and adrenal steroids (Martial et al., 1977; Tannenbaum et al., 1978; Evans et al., 1982; Wehrenberg et al., 1983; Jansson et al., 1984a) all influence GH release. GH secretion is characterised by a pulsatile rhythm in all mammalian species (Jansson et al., 1985a). In the rat, this pattern is age and sex-specific. Whereas GH secretion is similar in both sexes up to the age of 22 days, it differs by 30 days of age. Between 25 and 30 days of age, growth hormone (GH) levels increase leading to similar mean plasma GH levels in male and female rats (Eden, 1979). However in adult male rats, GH secretion is characterised by regularly occurring high amplitude peaks of release every 3-4 hours, thus corresponding to approximately 7 surges per day (Tannenbaum & Martin, 1976). In contrast, females typically exhibit a higher frequency, lower amplitude pattern of secretion (1-2 pulses every 2 hours). Therefore the trough period between GH pulses is longer in males than females and often falls below the limits of detection in males (<1ng/ml in males versus >5ng/ml in females) (Eden, 1979; Tannenbaum & Martin, 1976) (Fig. 1.11). The most obvious manifestation of the different patterns of GH secretion, is the different growth rates in male and female rats which, like GH release, are similar up to 22 days of age, but by 30 days of age, male rats show a characteristic increase in weight gain (Jansson et al., 1982a; 1982b).

### 1.4.1 Hypothalamic Control of GH Secretion

The hypothalamus has both stimulatory and inhibitory effects on GH secretion from the anterior pituitary (Kurlich et al., 1968). The hypothalamus predominantly stimulates GH secretion through GRF release from the ventromedial nucleus and arcuate nucleus into the median eminence (Frohman & Bernardis, 1968; Martin et al., 1974; Millard et al., 1982). Electrolytic lesions of either the ventromedial nucleus or the arcuate nucleus

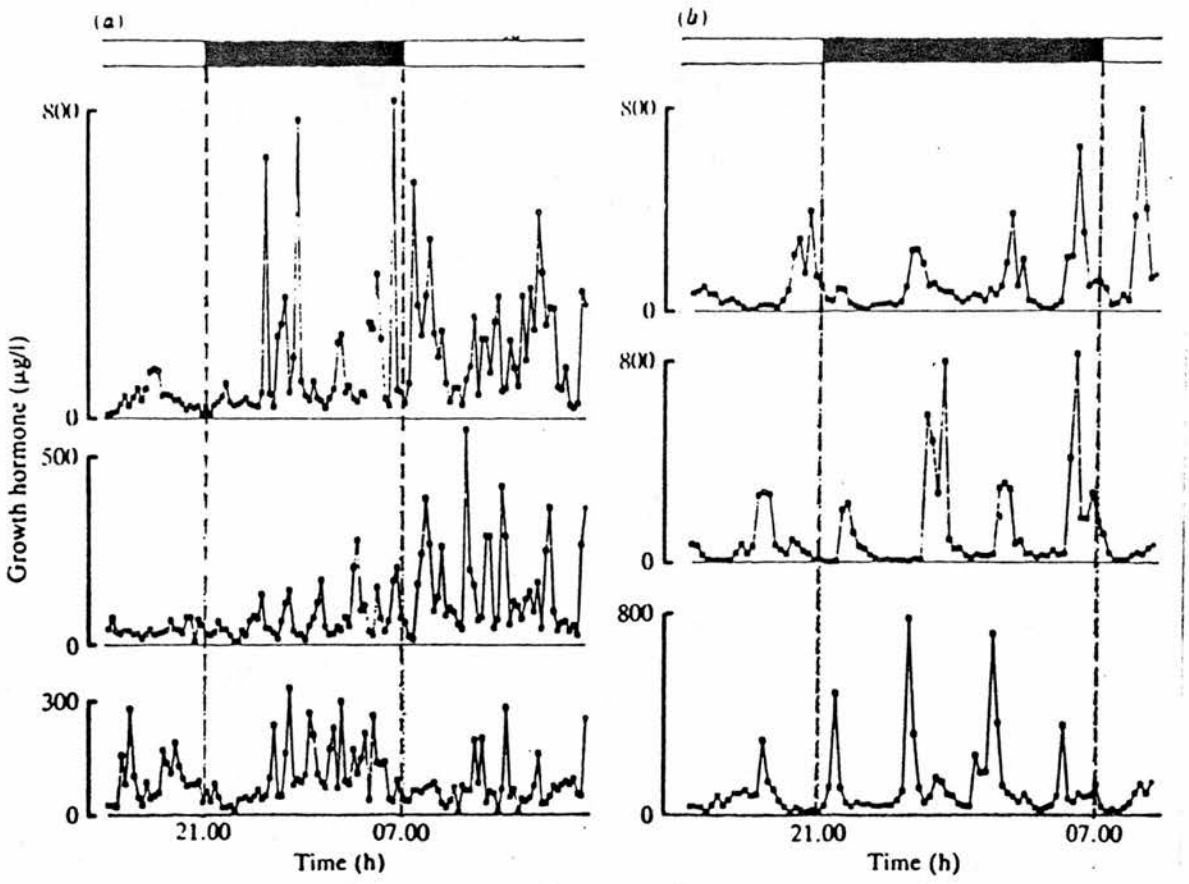



**Figure 1.11:**

Plasma concentrations of GH in three (a) adult female and three (b) adult male rats over 24h. The period of darkness was from 2100-0100h (solid bar). The y-axis is different for the two groups. Taken from Clark et al., 1986.

leads to decreased pituitary GH levels and suppressed pulsatile GH secretion (Frohman & Bernardis, 1968; Martin et al., 1974). In addition electrical stimulation of the two hypothalamic areas results in induction of GH secretion (Martin et al., 1978; Frohman et al., 1968). GH secretion is predominantly inhibited through SS release from the periventricular preoptic/anterior hypothalamic area into the median eminence (Alpert et al., 1976). Low plasma trough levels of GH are probably maintained by SS release. SS antibodies increase basal GH levels in male rats (Ferland et al., 1976; Terry & Martin, 1981), and depletion of hypothalamic SS stores by electrical lesioning of the preoptic area increase trough GH levels and cause more frequent pulses of GH release (Willoughby & Martin, 1978). However, the effects of SS on the amplitude of GH pulses are not clear, with some investigators reporting increased pulse amplitude following administration of SS antibodies (Ferland et al., 1976), while this effect is not reproduced by others (Terry & Martin, 1981).

SS-induced inhibition of GH release from rat pituitaries *in vitro* is followed by rebound release of GH after withdrawal of SS (Carlson et al., 1974; Stachura, 1976). This effect is mimicked *in vivo* by administration of SS antibodies in rats (Tannenbaum et al., 1978; Eden et al., 1981; Tannenbaum, 1981), suggesting that the pulsatility of GH *in vivo* is due at least in part to the pulsatile removal of SS. However administration of SS antibodies to rats with electrolytic lesions to GRF containing neurones in the ventromedial hypothalamus and arcuate nucleus, although increasing baseline GH levels, does not restore the pulsatility of GH secretion (Millard et al., 1982; Eiklebloom & Tannenbaum, 1983). In contrast, exogenous GRF treatment of rats with the same electrolytic lesions did induce GH pulsatility (Tannenbaum et al., 1983), and GRF antibodies have been shown to abolish GH pulsatility in male rats (Wehrenberg et al., 1982). Thus spontaneous GH pulsatility may be dependent on GRF release from the hypothalamus.

### 1.4.2 Feedback Regulation of GH Secretion

GH itself has a role in regulating its own secretion through a short-loop feedback mechanism. Exogenous GH administration leads to a reduction in hypophyseal GH content and amplitude of endogenous plasma GH pulses in rats (Krulich & McCann, 1966; Willoughby et al., 1980; Abe et al., 1983). The concentration of GH in the pituitary is reduced by implanting GH in the median eminence (Katz et al., 1969), and endogenous GH pulse amplitude is decreased after administration of GH into the lateral cerebral ventricle (Abe et al., 1983; Tannenbaum, 1980) suggesting that feedback regulation of GH may involve the hypothalamus. GH autoregulation at the hypothalamic level appears to involve SS. SS levels in the median eminence are reduced in

hypophysectomised rats, but this can be restored to normal by GH replacement (Hoffman & Baker, 1977; Patel, 1979). In addition GH administration to normal rats leads to increased hypothalamic SS content (Patel, 1979).

### 1.4.3 Other Regulators of GH Secretion

Many other factors appear to be involved in the regulation of GH secretion from the anterior pituitary including glucocorticoids, thyroid hormone, monoamines and various neuropeptides. The regulatory actions of these factors are reviewed in detail in Jansson et al. (1985a).

Monoamines are present in the hypothalamus in high concentrations, and appear to induce GH release by stimulating GRF release from the hypothalamus via activation of $\alpha_2$ adrenergic receptors. In addition, endogenous opioids such as ß-endorphin, enkephalin and dynorphin induce GH release *in vivo*, but this does not appear to be a direct effect on somatatrophs, and may reflect GRF release as a result of $\alpha$-adrenergic stimulation.

Thyroid hormones play an important role in the control of GH synthesis and secretion, such that thyroidectomy leads to a decrease in pituitary GH content and abolishes GH pulsatility in male rats, while thyroid replacement reverses these effects (Solomon & Greep, 1959; Coiro et al., 1979; Suzuki et al., 1980). Thyroid hormones and glucocorticoids have synergistic stimulatory actions on the activity and mRNA levels of GH in cultured pituitary cells (Martial et al., 1977). In addition, glucocorticoids and thyroid hormone stimulate GH gene expression leading to increased levels of GH mRNA in cultured pituitary cells (Evans et al., 1982). Glucocorticoids appear to stimulate transcription of the GH gene, resulting in increased accumulation of GH mRNA (Evans et al., 1982). In addition, *in vivo* glucocorticoid manipulation may enhance the pituitary GH response to GRF (Wehrenberg et al., 1983).

### 1.4.4 Sexual Dimorphism of GH Secretion

There is a striking sex difference in the pattern of GH secretion in adult rats (Saunders et al., 1976; Eden, 1979) as previously mentioned. Thus, in males, GH is secreted as high amplitude pulses every 3-4h, interspersed with low or undetectable levels between peaks, whereas female rats exhibit a more continuous pattern of secretion. Gonadal steroids have been implicated in the production and maintenance of this sexual dimorphism. For example, gonadectomy of male rats before puberty results in raised basal GH levels, and this effect can be reversed by testosterone replacement (Jansson et al., 1984a). In addition, oestrogens appear to elevate basal GH levels in male rats

(Jansson et al., 1983). Gonadectomy of female rats results in partially decreased basal GH levels, indicating the importance of androgens in the maintenance of low inter-pulse GH levels in male rats (Jansson et al., 1984a). The role of androgens in maintenance of the amplitude of GH release is less clear since gonadectomy pre-pubertally has no apparent effect on GH pulse amplitude of male rats (Jansson et al., 1984b), and in addition testosterone replacement at high supraphysiological levels does not affect the pulse amplitude (Jansson et al., 1985a). However, neonatally ovariectomised female rats treated with testosterone appear to exhibit high amplitude GH pulses in adult life (Jansson et al., 1985b). In contrast, male rats gonadectomised neonatally show a decrease in GH peak amplitude and increased GH trough levels compared to normal males (Jansson et al., 1985b; Jansson & Frohman, 1987), and this effect can be reversed by testosterone replacement (Jansson & Frohman, 1987). However neonatal testosterone replacement although restoring the high amplitude of GH pulses, led to increased frequency, and shortened duration of these pulses (Jansson & Frohman, 1987). If however neonatal testosterone replacement is continued into adulthood, the GH secretory pattern was found to be indistinguishable from normal males. This therefore suggests that adult androgen secretion plays both a corrective and a maintenance role in the sexual dimorphism of GH release. During puberty, oestradiol administration to male rats appears to decrease GH peak amplitude, while ovariectomy of the females leads to increased peak amplitudes (Jansson et al, 1984a). No such effects of ovariectomy or oestradiol replacement have been observed in postpubertal rats (Jansson et al., 1984a).

Autoradiographic studies by Herbert & Sheridan (1983) have demonstrated that hypophyseal somatatrophs have oestrogen binding sites suggesting the possibility of a direct mechanism of action of oestrogens on somatatrophs. In addition, treatment of ovariectomised female rats with [$^3$H] oestradiol for 1h, showed that oestradiol was loclised in the nuclei of 80-90% of GH-secreting cells (Shirazu et al., 1990). The effects of oestrogens on GH release from pituitary cells *in vitro* are conflicting. Thus it has been shown that oestrogens increase (Komolov et al., 1980), decrease (Birge et al., 1967) or have no effect (Webb et al., 1983) on GH secretion. In contrast there are few reports of testosterone effects on GH release *in vitro*. It has however been recently shown that the effect of gonadal steroids on anterior pituitary cells isolated from rats gonadectomised before puberty, depends on the sex of the source. Thus the GH response to GRF and SS was increased by testosterone only in male pituitary cells, and was decreased by oestrogen only in female pituitary cells (Hertz et al., 1989), suggesting that the sex differences present before puberty were maintained *in vitro* for the period of the study.

### 1.4.5 Gonadal Steroids and GRF and SS Secretion

To date, there is no direct evidence demonstrating differences in GRF release between male and female rats. However indirect evidence from monosodium glutamate-treated rats (Maiter et al., 1991a), and passively immunised rats (Wehrenberg et al., 1982; Ono et al., 1991; Painson & Tannenbaum, 1991) suggests that baseline GRF secretion is slightly elevated with low amplitude GRF pulses in female rats, and that baseline GRF values are lower with higher amplitude pulses in male animals. In addition, GRF mRNA levels are 2-3 times higher in adult male than female rat hypothalami (Maiter et al., 1991b; Mizobuchi et al., 1991). The effects of gonadal steroids in the maintenance of this putative sexual dimorphism are not clear. It has been shown that GRF mRNA levels are not affected by gonadectomy or sex steroid replacement in adult male or female rats (Maiter et al., 1991b; de Gennaro et al., 1989). However it has also been reported that male hypothalamic GRF mRNA content is decreased following castration, and this effect can be reversed by testosterone replacement (Zeitler et al., 1990). In addition, 1h after injection of ovariectomised feamle Sprague-Dawley rats with [$^3$H] oestradiol, the tracer was found in 35% of GRF-producing cells (Shirasu et al., 1990).

Similarly to GRF, there is no direct evidence demonstrating a sexually dimorphic pattern of SS release. However, conscious freely moving female rats consistently respond to GRF administration, whereas male rats only respond intermitently, suggesting that hypothalamic release of SS is different between male and female rats (Clark & Robinson, 1985). Several other studies have also suggested differences in SS release between male and female rats (Wehrenberg et al., 1982; Ono, et al., 1991; Painson & Tannenbaum, 1991). Gonadectomised male rats have been shown to express lower levels of SS mRNA in the periventricular nucleus compared to controls (Werner et al., 1988; Zorrilla et al., 1990). This effect was reversed by testosterone replacement, while oestradiol treatment had no effect in male animals (Argente et al., 1990). Similarly in female rats, ovariectomy has been shown to decrease SS mRNA levels in the periventricular nucleus while oestradiol reverses this effect (Werner et al., 1988; Zorrilla et al., 1990). These results suggest that sex steroids exert their effects on SS at the level of transcription, and that a reduction in the sex steroid environment leads to similar effects on SS in male and female rats. Whether gonadal steroids have direct or indirect effects on SS is not known, but since it has recently been demonstrated that there is no oestrogen response element on the SS gene (Goodman et al., 1990), a direct action of sex steroids is unlikely.

### 1.4.6 Physiological Significance of Sexually Dimorphic Patterns of GH

The most obvious manifestation of the differences in GH secretion is seen in the different growth rates of male and female rats which diverge at puberty leading to faster growth rates in the male animal (Wehrenberg & Giustina, 1992). In addition, hypophysectomised rats treated with GH showed increased growth rates when treated with pulsatile GH compared to continuous GH (Jansson et al., 1982a; Clark et al., 1985). However when administration frequency is increased, growth rates decrease indicating that high amplitude and low baseline levels of GH (ie male GH patterns) induce higher rates of somatic growth than low peak/high trough plasma levels of GH (ie female GH patterns).

Rat liver expresses several drug and steroid metabolising enzymes which are expressed in a sexually dimorphic manner (Gustafsson et al., 1983). For example two cytochrome P450 enzymes, 16α-hydroxylase and androstanediol disulphate 15ß-hydroxylase, exhibit sexually differentiated expression in rat liver (Morgan & Gustafsson, 1987). However, it has been demonstrated that this is not due to a direct effect of sex steroids since gonadal steroids have no effect on hepatic steroid metabolism in the absence of the pituitary (Kamatak et al., 1985). In contrast, GH administration to hypophysectomised rats can cause feminisation or masculinisation of liver steroid metabolism depending on its mode of administration which can be manipulated to mimic physiological male or female patterns of GH (Gustafsson et al., 1983). This suggests therefore that normal GH patterns mediate the sexually differentiated expression of hepatic steroid metabolising enzymes. GH patterns are also important for several other sexually differentiated functions such as body growth (Eden et al., 1987), serum levels of apolipoprotein E and cholesterol (Oscarsson et al., 1989), CBG (Eden et al., 1987), and major urinary proteins in mice (Knopf et al., 1983; Norstedt & Palmiter, 1984).

3ß-hydroxysteroid dehydrogenase, which is a member of the short chain alcohol dehydrogenase superfamily (section 1.3.4), is expressed at higher levels in male rat liver due to testosterone induction of the enzyme in male animals which can be reversed by high constant (female pattern) GH administration (Naville et al., 1991). Sexual dimorphism of 11ß-OHSD in liver has been previously reported (Lax et al., 1978), but the role of GH in the maintenance of this dimorphism is unknown.

## 1.5 Aims of The Thesis

The aim of the thesis was firstly to investigate the tissue specific regulation of 11ß-OHSD. Liver, kidney and hippocampus were chosen as representative tissues. Firstly, kidney is a classical mineralocorticoid responsive tissue where the physiological actions of 11ß-OHSD are well understood. In contrast, the role of 11ß-OHSD in liver is not clear, but since 11ß-OHSD activity is particularly high in liver, and this was the original source of the purified enzyme, it is likely that 11ß-OHSD has some importance in this glucocorticoid receptor-rich tissue. Finally hippocampus was chosen since it contains mineralocorticoid receptors and 11ß-OHSD, but in contrast to kidney, responds to physiological glucocorticoids *in vivo*.

Adrenal steroids have a wide range of actions in many tissues including liver, kidney and hippocampus. It is quite conceivable that 11ß-OHSD which regulates glucocorticoid levels *in vivo* could itself be regulated by both pharmacological glucocorticoid manipulations as well as chronically raised physiological glucocorticoids for example during chronic stress. These studies are discussed in Chapter 3.

That 11ß-OHSD activity in liver is expressed at higher levels in male than female animals is well established. In Chapter 4, gonadal steroid manipulations demonstrate that this sex difference is due to oestrogen repression of 11ß-OHSD in female liver. In addition, the role of sex-specific patterns of growth hormone secretion in the maintenance of this sexual dimorphism is considered in Chapter 5.

In light of recent literature suggesting the presence of a second gene encoding an isoform of 11ß-OHSD which is more likely to be responsible for the regulation of access of glucocorticoids to mineralocorticoid receptors in renal distal tubules and cortical collecting tubules, the final studies in this thesis were undertaken to determine whether the 'liver-type' 11ß-OHSD was capable of altering glucocorticoid access to GR when expressed in an intact mammalian cell system.

# CHAPTER 2

# MATERIALS AND METHODS

---

In general, all basic protocols used in these studies can be found in Sambrook *et al.* (1989).

## 2.1 DNA Analysis Techniques

### 2.1.1 Restriction Endonuclease Digestion and Electrophoresis of DNA

DNA was digested using 1 unit of restriction enzyme per μg of DNA in 1x restriction buffer which was supplied with the enzymes (as a 10x solution) and distilled $H_2O$ ($dH_2O$) to the required volume. Digestion of plasmid DNA was routinely carried out at 37°C for 1-2h. The enzyme reaction was stopped by heating digests to 60°C for 10 min and digested DNA was visualised after electrophoresis through agarose gels. 1% agarose gels (w/v) were routinely used to separate DNA fragments of 400bp-7kb and were prepared by dissolving agarose in 1x TBE (10x TBE = 108g Tris, 55g boric acid, 4.7g EDTA made up to 1 litre with $dH_2O$) in a microwave oven. The agarose solution was allowed to cool slightly before adding 1-5μl of ethidium bromide (10 mg/ml) and pouring the solution into a gel mould with a comb in place - the tooth size of the comb dependent on the size of DNA sample - and allowed to set. The gel was placed in a horizontal gel apparatus containing sufficient 1x TBE to cover the surface of the gel. 2μl of agarose loading buffer (0.25% (w/v) bromophenol blue, 0.25% xylene cyanol (w/v), 30% glycerol) was added to DNA samples before loading onto the gel. Gels were usually run at 50-80mA with a 1kb DNA ladder (Gibco BRL) containing DNA fragments of 75bp-12kb as a size marker, for as long as required to see sufficient separation of DNA fragments.

## 2.1.2 Subcloning of DNA Fragments

### 2.1.2.1 *Plasmid Vectors*

**p11DH:** This is a 4.2kb plasmid which was constructed using a 1.2kb EcoR1 fragment of the 11ß-OHSD cDNA (Agarwal et al., 1989) subcloned into pBluescript. pBluescript has the capability to maintain a high copy number and also contains the ampicillin resistance gene ß lactamase and an artificial polylinker inserted into the lac Z operon. pBluescript also contains the phage T3 and T7 RNA polymerase promoter sequences flanking the polylinker thus enabling *in vitro* synthesis of specific single stranded sense (T7) and antisense (T3) RNA transcripts.

**prMR:** The rat mineralocorticoid receptor plasmid was constructed by subcloning a 513bp EcoR1 fragment of the receptor cDNA into pGEM4 (Arriza et al., 1988). pGEM4 contains the ß-lactamase gene and an artificial polylinker inserted into the lac Z operon. It also contains the phage SP6 and T7 RNA polymerase transcription initiation sites flanking the polylinker. This therefore allows *in vitro* synthesis of specific single stranded sense (T7) and antisense (SP6) RNA transcripts.

**prGR:** The rat glucocorticoid receptor plasmid was constructed from a 674bp Pst1/EcoR1 fragment of the receptor subcloned into pGEM3 (Miesfeld et al., 1984). pGEM3 like pGEM4, contains the ß-lactamase gene and an artificial polylinker inserted into the lac Z operon. However in pGEM3, the polycloning site is in the opposite orientation to pGEM4 so that *in vitro* synthesis of a single stranded sense RNA transcript is obtained using the SP6 RNA polymerase, while an antisense transcript can be obtained from the T7 RNA polymerase transcription initiation site.

**p7S:** A plasmid vector containing the the ampicillin resistance gene and entire coding region of the 7S cDNA subcloned into pAT 153 (Balmain et al., 1982). 7S is a ribosomal RNA which is found in relative abundance in numerous cells (Balmain et al., 1982) and can therefore be used to control for RNA loading on northern analysis.

**pJ3:** A 3.5kb vector containing the ampicillin resistance gene and the SV40 (Simian Virus 40) origin and early promoter (Morgenstern & Land, 1990). This plasmid was used in transfection studies as a control for pSL1 activity.

**pSL1:** This plasmid was constructed by myself by subcloning a 1.2kb EcoR1/Sst1 fragment of p11DH into pJ3 (Morgenstern & Land, 1990) so that the entire coding region of 11ß-OHSD was under control of the SV40 promoter (section 2.1.2.3). This plasmid was used in transfection studies to encode the 11ß-OHSD protein.

**pKC275:** This plasmid was used in transfection studies as an internal control for transfection efficiency since it encodes the ß-galactosidase gene. pKC275 was constructed by Karen Chapman by subcloning the HindIII/Bam H1 fragment of ß-

galactosidase into pRSVCAT (Gorman et al., 1982) thus producing a ß-galactosidase gene under control of the Rous sarcoma virus promoter.

**pRShGR:** The entire coding sequence of human GR (hGR) is contained within this expression plasmid under control of the SV40 promoter (Giguere et al., 1986). The coding sequence of hGR was subcloned into pRSVCAT (Gorman et al., 1982) which had the CAT coding sequence removed, and the SV40 origin added. The resulting expression plasmid also contains the ß-lactamase gene encoding ampicillin resistance.

**MMTV-LTR luciferase:** This plasmid contains the ampicillin resistance gene and the entire coding sequence of firefly luciferase under control of the mouse mammary tumour virus long terminal repeat promoter (Lefebvre et al., 1991). The expression plasmid was constructed by subcloning the coding sequence of firefly luciferase into pMLuc which contains the MMTV-LTR promoter (Nordeen, 1988).

**RSV-luciferase:** contains the entire coding region of firefly luciferase under control of the Rous sarcoma virus promoter (de Wet et al., 1987). This expression plasmid was also subcloned into pMLuc (Nordeen, 1988) and in addition contains the ß-lactamase gene.

2.1.2.2 *Bacterial Media*

**LB (Luria-Bertoni)** broth per litre: 10g bactotryptone (Difco); 5g bacto yeast extract (Difco) and 10g NaCl. For LB agar, 15g agar was added per litre to LB broth.

**M9 Minimal Medium** per litre: 1x M9; 0.3% casamino acids; 0.5% glucose; 0.1mM $CaCl_2$; 0.00002% vitamin $B_1$ and 4mM $MgSO_4$.

**Media Additives**: Ampicillin (Sigma) at a concentration of 100µg/ml was used to select for plasmids containing ampicillin resistance.

All media were sterilised by autoclaving and media additives sterilised by filter sterilisation. Bacteria were grown at 37°C in a shaking incubator to provide aeriation.

2.1.2.3 *Construction of the pSL1 Subclone*

p11DH was digested EcoR1 and SSt1 and run on 1% low melting point agarose gels as described (section 2.1.1). A gel slice containing the 1.2kb fragment of p11DH was cut from the gel using a sterile scalpel, weighed, put in a 1.5ml eppendorf tube and dissolved in $dH_2O$ (2µl/mg) at 70°C for 5-10 min. The DNA was cleaned by adding 100µl Tris-saturated phenol, vortexing and centrifuging for 10 min. in a bench top eppendorf centrifuge. The upper aqueous phase containing the DNA was removed into a fresh eppendorf tube and was extracted twice; once with 100µl Tris-saturated phenol and 100µl chloroform:isoamylalcohol (24:1, v/v) and once with

100µl chloroform:isoamylalcohol. The DNA fragment was then ethanol precipitated with 0.15 volumes of 5M NaCl or 0.05 volumes of 8M ammonium acetate, and 2.5 volumes of ethanol, put on dry ice for 5-10 min. and then centrifuged for 10 min. The supernatant was discarded and the DNA pellet dried briefly at room temperature before resuspension in an appropriate volume of TE (10mM Tris-HCl (pH 8), 0.1M EDTA). Recovery of precipitated DNA was checked by electrophoresis of an aliquot (1-2µl) of the total recovered DNA, through an agarose gel.

10µg of pJ3 was linearised with EcoR1/Sst1 (10 units) in a 20µl reaction containing 2µl of 10x restriction buffer, at 37°C for 1-2h. An aliquot of the restriction digest was run on an agarose gel to check the concentration of linearised vector. The reaction was diluted 2-fold to reduce the salt concentration; then the digested pJ3 was treated with calf intestinal phosphatase (CIP) (1U/µl) at 37°C for 1h to prevent religation of the vector with itself.

The p11DH restriction fragment was inserted into pJ3 in a 10µl ligation reaction containing 1µl of 10x ligation buffer (50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 1mM DTT), 1mM ATP (pH 7.0), 0.5 units T4 DNA ligase and a 1:3 molar ratio of linearised pJ3:p11DH fragment. Ligation reactions were incubated at 37°C for 1-2h.

2.1.2.4 *Bacterial Transformation*

*Escherichia coli* strain HB101 were made competent for DNA uptake by the $CaCl_2$ method (Sambrook *et al.*, 1989). Cells were grown in 100ml LB to mid log phase ($A_{600}$ = 0.3-0.6) then harvested at 3000rpm in a Beckman J20 rotor for 10 min. at 4°C. The pellet was resuspended in 10ml ice-cold 0.1M $CaCl_2$ and stored on ice for 10 min. Cells were then repelleted, and resuspended in 2ml 0.1M $CaCl_2$ and could then be stored at 4°C for up to 4d before transformation. 150µl of competent cells were mixed with up to 50ng DNA in a volume of 10µl TE and stored on ice for 10 min. Cells were heat shocked at 42°C for exactly 90 sec and then transferred back onto ice. The entire transformation mix was spread onto LB agar plates containing 100µg/ml ampicillin using a glass spreader. Plates were incubated at 37°C overnight. Control plates for transformations included HB101 alone (which do not have the ampicillin resistance gene) and plasmids which transform well e.g. pGEM3. Cells which grew on the selective media represented cells which had successfully been transformed by the recombinant DNA bearing the ampicillin resistance marker. Plates with transformed colonies were stored at 4°C for up to 3 months.

## 2.1.3 DNA Preparation

### 2.1.3.1 *Plasmid DNA Minipreparation*

A single transformed bacterial colony was transferred into 3ml LB containing 100μg/ml ampicillin and incubated overnight in a loosely capped tube at 37°C. 1.5 ml of the overnight culture was recovered by centrifugation at 14,000 rpm in a bench top eppendorf centrifuge for 1 min (the rest of the culture was stored at 4°C). The pellet was resuspended in 100μl ice cold GTE (50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA) then mixed gently with 200μl of freshly prepared alkaline-SDS solution (0.2M NaOH, 1% SDS) and stored on ice for 5-10min. Ice cold potassium acetate, 150μl (3M with respect to potassium, 5M with respect to acetate) was added and mixed by inverting the tube several times before storing on ice for 5-10 min. Denatured chromosomal DNA and cellular proteins were removed by centrifugation for 5 min. The supernatant was transferred to a fresh eppendorf tube and extracted with an equal volume of phenol:chloroform/isoamyl alcohol. After mixing and centrifuging for 2 min, the supernatant was transferred to a fresh eppendorf tube, and the DNA precipitated with 2 volumes of ethanol at room temperature for 5 min. After centrifuging for 5 min, the supernatant was aspirated and the DNA pellet rinsed with 70% ethanol. The pellet was then dried briefly at room temperature and resuspended in 50μl TE containing DNase-free RNase (20μg/ml) and stored at -20°C.

### 2.1.3.2 *Large Scale Plasmid DNA Preparation*

A single bacterial colony was transferred asceptically into 3ml LB containing 100μg/ml ampicillin and incubated overnight in a loosely capped tube at 37°C. The overnight culture was diluted in 500ml LB or M9 minimal medium containing 100μg/ml ampicillin, and grown in a 2 litre flask at 37°C overnight, then centrifuged at 6000rpm for 5 min at 4°C in a Beckman J2-MC centrifuge. The bacterial pellet was resuspended in 12ml of ice-cold GTE solution (50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA) and mixed immediately with 24ml of freshly prepared alkaline-SDS solution (0.2M NaOH, 1% SDS), then stored on ice for 5 min. 16ml of ice-cold potassium acetate (3M with respect to potassium, 5M with respect to acetate) was added, mixed well, and stored on ice for a further 10 min. before centrifuging at 6000rpm for 10 min. at 4°C. The supernatant was strained through 4 layers of sterile gauze into 250ml centrifuge pots, and precipitated at room temperature for 20-30 min. by the addition of 32ml isopropanol. Plasmid DNA was recovered by centrifuging at 10,000rpm for 5min. at 4°C. The DNA pellet was dried at room temperature before being resuspended in 1.5ml TE buffer. Following resuspension, 1g of CsCl per ml of DNA solution was added and dissolved in the DNA by gentle

warming at 30°C. Then 50µl of ethidium bromide (10mg/ml) was added, and the resulting DNA/CsCl solution transferred into a 3ml Beckman Quick Seal ultracentrifuge tube with enough CsCl solution (prepared by adding 100g CsCl to 100ml TE) to fill the tubes, and centrifuged at 70,000rpm for 17h at 20°C in a Beckman Optima LTX 120,000rpm Ultracentrifuge. Plasmid DNA bands were collected through the tube wall using a syringe and a 21 gauge needle. For the purposes of transfections, plasmid DNA was transferred to fresh heat seal tubes with enough CsCl solution to fill the tubes, and centrifuged again for 4h at 100,000rpm or 17h at 70,000rpm at 20°C. For all other purposes, a single centrifugation was sufficient to purify the DNA. After collecting the DNA band through the wall of the tube, ethidium bromide was removed from the plasmid DNA by extracting several times with equal volumes of water saturated butanol (until both aqueous and organic phases were no longer pink). The plasmid DNA was precipitated in 2 volumes of 70% ethanol for 10 min. at room temperature, and recovered by centrifugation at 14,000rpm in a bench top eppendorf centrifuge. The DNA pellet was then dried briefly at room temperature and resuspended in an appropriate volume of TE.

The concentration and purity of recovered plasmid was determined spectrophotometrically by measuring absorbance at 260nm and 280nm in a Shimadzu UV-160A spectrophotometer. Plasmid DNA was routinely stored at -20°C.

Occasionally, if the plasmid to be prepared did not have a high copy number, or if it was required in larger amounts than are normally obtained from large scale DNA preparations, chloramphenicol amplification of the plasmid was employed. After diluting the 3ml overnight culture into 500ml in a 2 litre flask, the preparation was allowed to grow until the absorbance of the culture at 600nm was 0.6-0.8. At this point, 2.5ml of 34mg/ml chloramphenicol in ethanol was added to each culture. Chloramphenicol is an antibiotic which inhibits bacterial replication but not DNA amplification. Therefore more of the DNA produced from this kind of preparation is the DNA of interest and less is the bacterial DNA.

## 2.2 Preparation Of Nucleic Acid Probes

### 2.2.1 Random Priming of DNA

This method of random priming of DNA was developed by Feinberg and Vogelstein (1983) and is based on the hybridisation of a mixture of all possible hexanucleotides to the DNA to be labelled. The complementary strand is then hybridised from the 3' OH termini of the hexanucleotide primers using Klenow

enzyme in the presence of radiolabelled nucleotides using a Random Primed DNA labelling Kit (Boehringer Mannheim).

p11DH plasmid DNA was digested with EcoR1 (section 2.1.1) and the resulting 1.2 kb 11ß-OHSD fragment isolated on a 1% low melting point agarose gel (section 2.1.2.4) and the recovery checked on a 1% agarose gel. The DNA was then denatured at 95°C for 10 min and subsequently cooled on ice. The reaction mix (made up on ice) consisted of up to 25ng denatured DNA; 3µl dATP, dGTP, dTTP mixture (prepared by making a 1+1+1 mixture of the nucleotides); 2µl hexanucleotide primer mixture; 5µl [$\alpha^{32}$P] dCTP (3000 Ci/mmol; Amersham International, UK) made up to 19µl with $dH_2O$ and 1µl Klenow enzyme. The reaction was incubated at 37°C for 1h, then terminated by adding 2µl 250mM EDTA (pH 8.0). The DNA probe was precipitated by adding 1µl 5M NaCl, 1µl glycogen and 65µl ice-cold ethanol and storing on dry ice for 5 min. The pellet was recovered by centrifuging at 14,000rpm for 10 min in a bench top eppendorf centrifuge, the supernatant discarded and the pellet dried and resuspended in 50µl of $dH_2O$.

7S plasmid DNA was also linearised with EcoR1 but was not routinely isolated from agarose gels. It was however, labelled in exactly the same way as the 11ß-OHSD fragment.

### 2.2.2 Synthesis of Radioactive RNA Probes

RNA probes were synthesised *in vitro* using phage T3, T7 or SP6 RNA polymerase systems for *in situ* hybridisation experiments.

Rat MR, GR and 11ß-OHSD plasmids were linearised with restriction enzymes distal to the insert (Table 2.1) to produce both sense and antisense DNA templates as necessary. 0.5-1.0µg of linear DNA template in a DEPC-treated eppendorf was incubated with 2µl of 5x transcription buffer (5x TB= 200mM Tris-HCl (pH 7.5), 30mM $MgCl_2$, 10mM spermidine, 50mM NaCl); 1.5µl of a 10mM ATP, 10mM CTP, 10mM GTP mix; 0.5µl of 200mM dithiothreitol (DTT); <0.3µl of RNase Inhibitor (Promega); 1.2µl of 175µM UTP; 1µl of $^{35}$S UTP (40mCi/ml, Amersham International; UK) and 1µl of the appropriate RNA polymerase in a total volume of 10µl with DEPC-treated $H_2O$ at 37°C (40°C for SP6) for 1h. The volume of the reaction was then increased by adding 2µl 5x transcription buffer and 8µl DEPC-treated $H_2O$, and the DNA template degraded using 1µl RNase-free DNase at 37°C for 10 min. The probe was purified by phenol:chloroform extraction (10µl each of phenol and chloroform:isoamyl; 24:1), and after centrifugation in a bench top centrifuge, the upper aqueous layer was removed into a fresh DEPC-treated eppendorf, and the probe precipitated with 1µl glycogen, 1µl of 10M ammonium

acetate and 50µl of ethanol on dry ice for 5 min. The pellet was recovered by centrifugation for 15 min, dried briefly at room temperature and resuspended in 50µl of DEPC-treated $H_2O$. Routinely, 1µl of the resuspended probe was counted in 5ml Cocktail T (BDH) to check the incorporation of $^{35}S$ UTP into the probe, and 1µl run on a vertical 4% polyacrylamide gel to check the integrity of the probe. The probe was then added to 500µl of 2x hybridisation buffer (1.2M NaCl, 20mM Tris-HCl (pH 7.5), 0.04% ficoll, 0.04% polyvinylpyrrolidone, 0.2% BSA, 2mM EDTA, 0.2mg/ml denatured salmon sperm DNA, 0.4g/ml dextran sulphate and 0.1mg/ml total yeast RNA made up in DEPC-treated $H_2O$) and stored at -20°C for no more than 3-4d since degradation of the probe appears to occur quite rapidly.

To check the incorporation of radionucleotide into the cRNA probes, 1µl of each probe was run on a 4% polyacrylamide 15cmx 17cmx 1.5mm gel. For a gel of this size, 10.5g of urea was dissolved in 3.3ml of 30% acrylamide and 2.5ml of 10x TBE, then made up to a total volume of 25ml. After dissolving, 250µl 10% ammonium persulphate and 25µl of TEMED were then added and the solution immediately poured into a 15cmx 17cmx 1.5mm glass sandwich which had been previously sealed with 1% (w/v) agarose (in $dH_2O$). A 20 tooth comb was placed in the top of the gel between the glass plates. After polymerisation, the gel plates were attached to vertical glass electrophoresis apparatus containing 1x TBE buffer. After adding 2µl loading buffer to the sample of probe, the probe was loaded onto the gel which was then electrophoresed at 40-80mA until the first dye front (bromophenol blue which runs at approximately 300bp) reached the bottom of the glass plates. One of the glass plates was then carefully removed leaving the gel on the other plate. The gel was covered with clingfilm and exposed to autoradiographic film (Hyperfim ß-max) overnight at room temperature before being developed in D19 developer for 2min. and fixed in a 1:5 dilution of Amfix, washed in tap water in the light and allowed to dry.

### 2.2.3 Synthesis of Non-Isotopic RNA Probes

Non-isotopic RNA probes were synthesised in a similar way to isotopic probes but instead of incorporating $^{35}S$-labelled UTP, biotin-11-UTP (biotin linked to UTP through an 11 nucleotide arm) was used as the label. Again 0.5-1.0µg linearised DNA was incubated in 1x transcription buffer, with 3µl of a nucleotide mix consisting of 10mM ATP, 10mM CTP and 10mM GTP; 1µl of 200mM DTT; 0.3µl of RNase Inhibitor; 2µl of 25µM UTP; 2µl of 10mM biotin-11-UTP and 1µl of the appropriate RNA polymerase in a total volume of 20µl with DEPC-treated $H_2O$ at 37°C (40°C for SP6) for 1h. The DNA template was degraded, purified and

| Plasmid | Purpose | Restriction Endonuclease |
|---|---|---|
| p11-DH | Template for antisense ISH probes | Sty I |
| | Template for sense ISH probes | Hind III |
| | Template for antisense northern probes | EcoRI |
| p7S | Template for antisense northern probes | EcoRI |
| prGR | Template for antisense ISH probes | Ava I |
| | Template for sense ISH probes | EcoRI |
| prMR | Template for antisense ISH probes | Hind III |
| | Template for sense ISH probes | EcoRI |

**Table 2.1:**
Restriction enzymes routinely used in linearising and isolating fragments of DNA from plasmids.

precipitated in the same way as the isotopic RNA probes (section 2.2.2). Biotinylated cRNA probes were routinely stored in DEPC-treated $H_2O$ at -20°C where they appeared to be more stable than isotopic probes and were used up to months after preparation. To check the integrity and incorporation of biotin label into the probes, a 1µl aliquot was run on a 1% denaturing agarose gel and blotted onto nitrocellulose or nylon membrane (section 2.3.1.3). Once dried, the blot was stained for the presence of biotin label using a BluGENE Nonradioactive Nucleic Acid Detection System (Gibco BRL). The membrane was rehydrated in buffer 1 (0.1M Tris-HCl (pH 7.5), 0.15M NaCl) at room temperature and then incubated in buffer 2 (3% bovine serum albumin (w/v) in buffer 1) for 1h at 65°C. The membrane was then incubated in a streptavidin-alkaline phosphatase conjugate for 10 min at room temperature with gentle agitation before washing twice in buffer 1 for 15 min. each. Following a wash in buffer 3 (0.1M Tris-HCl (pH 9.5), 0.1M NaCl, 50mM $MgCl_2$), the filter was incubated in a dye solution consisting of NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) for 30 min to 3h in a sealed hybridisation bag in the dark. When the colour reaction was sufficient, filters were washed in a 'stop buffer' (20mM Tris-HCl (pH 7.5), 0.5mM $Na_2EDTA$) to terminate the colour

development. Filters were baked in the oven at 80°C for 2h and stored protected from the light.

## 2.3 RNA Analysis Techniques

### 2.3.1 Northern Analysis

#### 2.3.1.1 *Tissue Dissection*

Rats were killed by decapitation and the organs of interest were rapidly removed, dissected on ice and immediately frozen down on dry ice. Hippocampus was dissected as follows: the brain was removed from the skull and the cerebellum severed from the cerebrum. The remainder of the brain was split into two halves down the midsaggital plane, the fornix detached and the hippocampus rolled away from the wall of the lateral ventricle. All tissues were frozen immediately on dry ice and stored at -70°C until required.

#### 2.3.1.2 *Extraction of RNA From Rat Tissues*

Total RNA was extracted from hippocampus, liver and kidney by the guanidinium thiocyanate method as described by Chomczynski and Sacchi (1987). Tissues were homogenised in 4M guanidinium thiocyanate, 0.025M sodium citrate, 0.5% sarcosyl and 0.1M ß-mercaptoethanol using sterilised Dounce tissue grinders. Routinely, half of the hippocampus was homogenised in 500µl of this denaturing solution, while liver and kidney were homogenised in 5-10ml of solution per g of tissue. Homogenised tissue was split into 500µl aliquots in DEPC-treated eppendorfs. DNA was precipitated by addition of 50µl 0.2M sodium acetate (pH 4) and protein was removed by 500µl water-saturated or citrate-saturated phenol and 100µl chloroform:isoamyl alcohol (49:1) extraction. The homogenates were vortexed vigorously and stored on ice for 15 min before centrifugation at 14,000 rpm in a bench top centrifuge for 20 min. The upper aqueous phase containing RNA was removed into fresh DEPC-treated eppendorfs, mixed with 200µl isopropanol to precipitate the RNA, and stored at -20°C for at least 1h. The RNA was centrifuged again at 14,000 rpm for 20min, and the RNA resuspended in 60µl guanidinium thiocyanate denaturing solution and 60µl isopropanol. The RNA was reprecipitated at -20°C for at least 1h, the pellet recovered by centrifugation for 10 min, washed in 100µl 70% ethanol, dried and resuspended in an appropriate volume of DEPC-treated $H_2O$. This was generally 20µl for hippocampal RNA and 50-100µl for liver and kidney RNA depending on the size of the RNA pellet. RNA concentration and purity

were estimated spectrophotometrically by measuring the absorbance at 260nm and 280nm, and aliquots stored at -70°C prior to use.

2.3.1.3 *RNA Electrophoresis and Capillary Transfer*

Aliquots of total RNA from hippocampus (15µg), liver (10µg) and kidney (10µg) were fractionated on 20x 25cm 1.2% agarose-2.2M formaldehyde denaturing gels. For 250ml gel mix, 3g of agarose was melted in 220ml DEPC-treated $dH_2O$ and after cooling slightly, 5ml formaldehyde and 25ml 10x MOPS buffer (10x MOPS= 0.2M MOPS (pH 7.0), 50mM NaAc, 5mM EDTA) were added and poured into the gel mould with a comb whose tooth size was appropriate to the amount of RNA to be loaded onto the gel. After setting, the gel was aged in the gel tank with 1x MOPS buffer. RNA samples were mixed with 50% deionised formamide, 12.5% formaldehyde and 12.5% 10x MOPS in a total volume of 20µl and denatured at 65°C for 15 min. 1µl of 10mg/ml ethidium bromide was added to 40µl of 2.5x loading buffer and 2µl of this mixture was added to each RNA sample. Samples were loaded onto the gel which was run either for 8h at 100V or for 16h at 50V. The gel was soaked in 20x SSC, then blotted onto nitrocellulose (Hybond C extra, Amersham International) or nylon (Hybond N, Amersham International) membrane by capillary transfer overnight. This was achieved by laying the gel on a transfer apparatus consisting of a wick of Whatman 3MM filter paper over an upturned gel mould running into a reservoir of 20x SSC. The nylon or nitrocellulose membrane, cut to exactly the same size as the gel was placed in direct contact with the gel. Two sheets of Whatman 3MM paper were placed over the membrane followed by paper towels to a depth of approximately 5cm. An even weight balanced on a glass plate was then placed on the apparatus and transfer allowed to take place. After checking the efficiency of transfer under UV, the membrane was baked between two sheets of Whatman 3MM paper at 80°C for 2h.

2.3.1.4 *Northern Hybridisation*

Northern hybridisation was routinely carried out by one of two methods:

(a) Nitrocellulose and nylon membranes were rehydrated in sterile 20x SSC and separated by layers of nylon mesh of the same size as the filter so that when the filters were rolled up, no part of the filters would be in direct contact. Filters and mesh were then rolled up together and inserted into a Hybaid Mini Hybridisation Oven bottle. Filters were prehybridised and hybridised in 20 ml of 50% formamide, 5x SSPE (20x SSPE= 6M NaCl, 0.18M $NaH_2PO_4$, 20mM EDTA; pH 7.4), 5x Denhardt's solution (0.1% BSA, 0.1% ficoll, 0.1% polyvinylpyrrolidone), 200µg/ml denatured herring testes DNA, 0.1% SDS and 1mM EDTA for at least 2h at 42°C while rotating in a

Note: aging the gel consists of soaking it in buffer in the gel tank.

Hybaid Mini Hybridisation Oven. Both prehybridisation and hybridisation buffer were made up fresh for each hybridisation. After prehybridisation, the random-primed $^{32}P$-labelled cDNA probe (section 2.2.1) was carefully added to the hybridisation bottle and the filters hybridised overnight at 42°C.

(b) Alternatively, membranes were prehybridised (for at least 2h) and hybridised (overnight) at 65°C in 10-20ml of 0.2M $NaH_2PO_4$, 0.6M $Na_2HPO_4$, 5mM EDTA, 6% SDS and 100µg/ml of denatured salmon sperm DNA. Hybridisation was carried out at 65°C overnight in the same way as above.

The next morning, the probe was carefully disposed of, and filters washed in the hybridisation bottle with approximately 50ml of 2x SSC (or 1x SSPE) and 0.1% SDS for 15-30 min at room temperature. For the following higher stringency washes, the filters were removed from the hybridisation bottles and placed in plastic containers of at least the same size as the filters containing 500ml of 0.2x SSC (or 0.1x SSPE) and 0.1% SDS. These containers were then sealed and incubated at 60°C for 30-60 min. on a rocking table to agitate the washing buffer. This wash was repeated once more, or until the radioactive signal reached background levels. Filters were then wrapped in clingfilm and exposed to Kodak XAR-5 film at -70°C at least overnight. Films were developed in Kodak D19 developing solution (Ilford) for 2 min, and fixed in a 1 in 5 dilution of Amfix fixative (Champion) for 2 min. All filters were stripped by submerging in boiling water which was then allowed to cool to room temperature. Stripping was checked by exposing filters to Kodak XAR-5 film overnight. Filters were then rehybridised with 7S in exactly the same way as above, to control for RNA loading. Both 7S and 11ß-OHSD were quantified by computer densitometry (Seescan, Cambridge, UK) and expressed as a ratio of the optical densities of 11ß-OHSD:7S.

### 2.3.2 *In Situ* Hybridisation Analysis

#### 2.3.2.1 *Tissue Preparation*

Rats were killed by decapitation and the brain rapidly removed, the cerebellum severed and the cerebrum frozen on dry ice. Tissue was stored at -70°C prior to sectioning in a cryostat at -20°C. 10µm coronal brain sections were cut and thaw-mounted onto slides which had been treated with gelatine and were poly-L-lysine coated. Sections were then stored at -70°C until required.

#### 2.3.2.2 *Isotopic In situ hybridisation*

Tissue sections were fixed in 4% paraformaldehyde/0.1M phosphate buffer (20mM $NaH_2PO_4$, 80mM $Na_2HPO_4$) for 10 min followed by three washes in 2x SSC

made up in DEPC-treated $H_2O$ for 5 min each. Both the fixation and washing treatments were carried out in sterilised glassware.

Sufficient hybridisation mix was made up to apply 200µl to each slide. The hybridisation mix consisted of 50% formamide, 10x $10^6$ counts/ml of radioactive cRNA probe, made up to a final volume with 2x hybridisation buffer. This hybridisation mix was denatured at 70°C for 10 min, cooled on ice and 10µl/ml of 1M DTT added. Tissue sections were dried around the edges with lens tissue before adding 200µl hybridisation mix to each slide and ensuring each tissue section was covered. The slides were placed flat in plastic boxes containing 3MM Whatman paper soaked with 'box buffer' (4x SSC, 50% formamide in DEPC-treated $H_2O$). These boxes were then sealed with autoclave tape and incubated at 50°C overnight. Following hybridisation, slides were rinsed twice in 2x SSC (made up in $dH_2O$) for 10 min at room temperature prior to RNase digestion with 200µl of RNase A (30µg/ml in 0.5M NaCl, 10mM Tris-HCl (pH 7.5), 1mM EDTA) at 37°C for 45-60 min. Slides were then washed in 2x SSC at room temperature for 1h followed by a more stringent wash of 0.1x SSC at 60°C for 1h. After dehydration in 50%, 70% and 90% ethanol in 0.3M ammonium acetate for 2 min each, slides were left to air dry overnight in a fume hood before being exposed to autoradiographic film (Hyperfilm ß-max, Amersham International, UK) for 10d or being dipped in photographic emulsion (K5, Ilford, UK) and stored at 4°C for 21-28d. Films were developed in D19 developer for 2 min and fixed in a 1:5 dilution of Amfix fixative for 2 min. Slides were developed for 3 min, rinsed in $dH_2O$, fixed for 3 min and washed 5x in $dH_2O$ for 5 min each in the light. In order to visualise silver grains clearly, slides were also counterstained with haematoxylin and eosin and coverslipped using DPX as a mountant.

Hybridisation with sense cRNA probes under the same conditions as above were performed as controls for isotopic *in situ* hybridisation.

2.3.2.3 *Non-Isotopic In Situ Hybridisation*

Non-isotopic RNA probes were added to the hybridisation mix at a concentration of 30µg/ml (determined spectrophotometrically). In all other aspects, non-isotopic *in situ* hybridisation was identical to the isotopic method.

Development of the non-isotopic *in situ* hybridisation was carried out with an ABC kit (Vector). The principle of this kit depends on the complexing of each biotin molecule with 4 avidin molecules. A complex of avidin-biotin-horseradish peroxidase (hence ABC) is therefore made and applied to the sections. Since MR, GR and 11ß-OHSD mRNA species are relatively rare, the system was amplified by incubating slides with a biotinylated anti-avidin antibody followed by another

incubation with the avidin-biotin-horseradish peroxidase complex. A substrate which turns brown (DAB) or red (AEC) in the presence of horseradish peroxidase is then added to indicate the presence of the original biotinylated probe.

After the stringent wash of 0.1x SSC at 60°C for 1h, sections were rinsed in 1x phosphate buffered saline (PBS) (1x PBS= 137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4$, 1.47mM $KH_2PO_4$) and incubated in the ABC complex for 30 min at room temperature. This was rinsed off briefly with 1x PBS, before addition of the biotinylated anti-avidin antibody (1:250 dilution) for 1h. Again slides were washed in 1x PBS and incubated with the ABC complex for 30 min. After a final wash in 1x PBS, sections were incubated in either 0.5mg/ml DAB (3,3'-diaminobenzidine) and 0.1% $H_2O_2$ in 1x PBS, or AEC (3-amino-9-ethylcarbazole; Vector kit) for 10 min. Slides were then rinsed in tap water, dehydrated (DAB), treated with methyl green as a counterstain and coverslipped using DPX as a fixative, or air dried (AEC; AEC is alcohol soluble) and coverslipped using an aqueous mountant.

Controls for non-isotopic *in situ* hybridisation included hybridisation of tissue sections with hybridisation mix minus biotinylated probes, and competition studies with 100-fold excess unlabelled homologous or heterologous probes added to the hybridisation mix.

#### 2.3.2.4 *Double In Situ Hybridisation*

Tissue sections were incubated in a hybridisation mix containing 10x $10^6$ counts/ml of the isotopic RNA probe and 30µg/ml of the biotinylated RNA probe at 50°C overnight, treated with 30µg/ml RNase A and washed to a maximum stringency of 0.1x SSC at 60°C (section 2.3.2.1). The biotin *in situ* hybridisation was then developed (section 2.3.2.2), the slides air-dried overnight and exposed to autoradiographic film or dipped in emulsion (section 2.3.2.1).

## 2.4 In Vitro Assays

### 2.4.1 11ß-OHSD Enzyme Assay

Rats were decapitated, liver, kidney and hippocampus removed and dissected on ice. Tissues were homogenised in Krebs-Ringer bicarbonate buffer (118mM NaCl, 3.8mM KCl, 1.19mM $KH_2PO_4$, 2.54mM $CaCl_2.2H_2O$, 1.19mM $MgSO_4.7H_2O$, 25mM $NaHCO_3$ and 0.2% glucose, pH 7.4, gassed with 95% $O_2$/5% $CO_2$ for 1h) in Dounce tissue homogenisers. Routinely, half a hippocampus was homogenised in 500µl Krebs-Ringer, while liver and kidney were homogenised in 5-10ml/g tissue.

After estimating the total protein concentration of each sample colorimetrically (Bio-Rad protein assay kit), duplicate aliquots of homogenate were incubated with 200μM NADP and 12nM 1,2,6,7 [$^3$H]-corticosterone ([$^3$H]-B) (specific activity approx. 84Ci/mmol, Amersham International) in a total volume of 250μl with Krebs-Ringer buffer (+ 0.2% BSA) at 37°C. Preliminary studies were carried out to optimise conditions for the enzyme assay and the protein concentration for each tissue was chosen such that (i) additional protein led to an approximately linear increase in percentage conversion of [$^3$H]-B to [$^3$H] 11-dehydrocorticosterone ([$^3$H]-A) in the presence of 200μM NADP and (ii) the percentage conversion was within the range of 20-50% conversion in a 10 min assay. This was found to be 50μg/ml liver, 25μg/ml kidney and 500μg/ml hippocampus (Fig. 2.1). After incubation, at least 2 volumes of ethyl acetate was added to each sample and mixed to extract the steroids from the homogenate. The upper aqueous layer was removed into fresh tubes and dried at 50°C under air. Steroids were resuspended in 100μl ethanol containing 2.5mg/ml each of unlabelled corticosterone and 11-dehydrocorticosterone and stored at 4°C overnight. 10μl samples of the steroids were pipetted onto silica gel coated aluminium thin layer chromatography plates (TLC, Merck Ltd), the plates allowed to dry, and another 10μl of the sample plated. This was repeated 4 times so that a total of 40μl of each steroid sample had been plated. TLC plates were divided into lanes of 2.5cm width to allow application of 8 steroid samples on each plate. Corticosterone and 11-dehydrocorticosterone were then separated by running the TLC plates in sealed tanks containing a mixture of chloroform: 95% ethanol (92:8). The steroid bands were visualised under ultra-violet light and scraped into scintillation vials containing 5ml liquid scintillant (Cocktail T, BDH). Radioactivity was quantitated on a ß-counter and the conversion of [$^3$H]-B to [$^3$H]-A was estimated from the radioactivity of each fraction.

### 2.4.2 Corticosterone Binding Globulin Assay

Trunk blood was collected from decapitated rats for both plasma corticosterone and corticosterone binding globulin (CBG) assays. Blood was collected into heparinised tubes, chilled immediately on ice, and centrifuged at 3000rpm for 10 min at 4°C. CBG levels were measured using the method of Martin et al. (1977). Endogenous steroids were removed from plasma by running the samples through sephadex LH-20 columns of 10 x 1cm. Plasma samples were then diluted 50:1 with TEGMD (30mM Tris, 1mM EDTA, 10% glycerol, 10mM sodium molybdate and 1mM dithiothreitol, pH 7.4). Aliquots of plasma were incubated overnight at 4°C with 200nM 1,2,6,7 [$^3$H]-corticosterone in TEGMD (total binding) or with 200nM

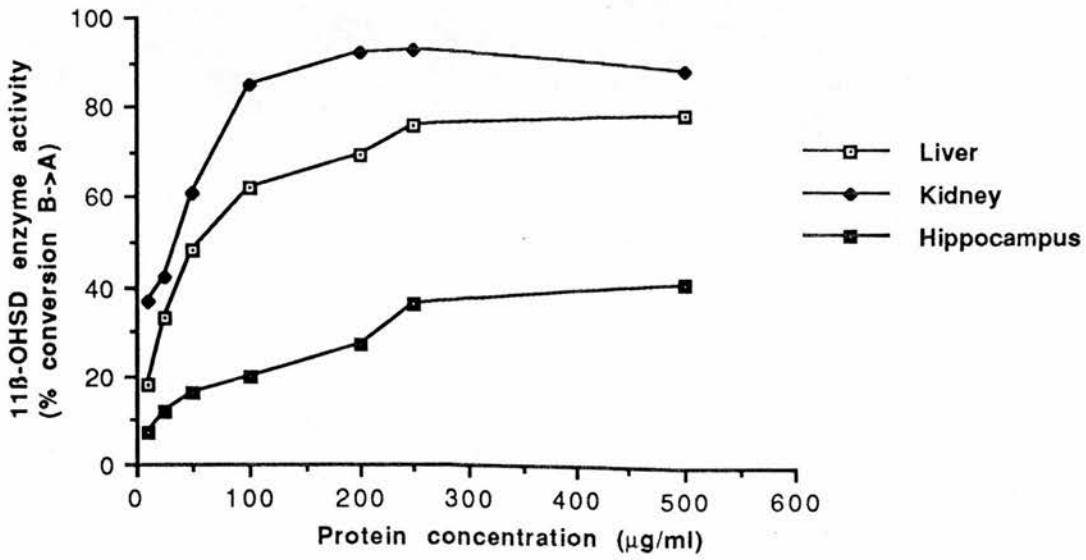



**Figure 2.1:**

11ß-OHSD activity with various protein concentrations of male rat liver, kidney and hippocampus. 11ß-OHSD activity is expressed as % conversion B->A in the presence of 200µM NADP at 37°C for 10 min. This is a representative experiment which was repeated three times. Each data point is the mean of duplicate measurements.

1,2,6,7 [$^3$H]-corticosterone and 0.1mM unlabelled corticosterone (non-specific binding). Separation of bound from free corticosterone was achieved by running samples through sephadex LH-20 columns as described above. The elutant from these columns was collected into scintillation vials containing 5ml Picoflour and counted on a ß-counter. CBG levels were expressed as pmol [$^3$H] corticosterone bound/mg protein.

### 2.4.3 Corticosterone Radioimmunoassay

Plasma corticosterone assays were performed on unextracted plasma samples as described previously (MacPhee et al., 1989). All plasma samples were diluted 1:25 (as well as 1:50 if the plasma was from potentially stressed rats) in 0.135M sodium borate buffer (0.133M boric acid, 67.5mM NaOH, 0.35% HCl (pH 7.4), supplemented with 0.5% BSA, 1.0% methanol and 0.1% ethylene glycol) and incubated at 75-80°C for 30 min, before being assayed in duplicate. Into 10x75mm borosilicate glass tubes, 50µl of diluted plasma sample was mixed with 100µl of a specific antibody against corticosterone raised in rabbits and 50µl [$^3$H] B. The antibody was diluted 1:5000, while the tracer was diluted 1:4500 (producing approximately 10,000cpm per tube), both in 0.135M sodium borate buffer. A standard curve of corticosterone was created by adding 1025µl 0.135M sodium borate buffer to 100µl stock corticosterone solution of 1µg/ml. This solution (12.8pmol/50µl) was then double diluted down to 0.00625pmol/50µl and assayed in duplicate in the same way as plasma samples. Standards and plasma samples were incubated overnight at 4°C before adding 500µl dextran-coated charcoal suspension (0.5% activated charcoal, 0.05% Dextran T70, 0.05% BSA in 65mM sodium borate buffer), vortexing and centrifuging at 3000xg for 30 min at 4°C to separate the antibody bound and unbound fractions. The bound fraction (supernatant) was counted in a ß-counter in 5ml picofluor (Picofluor 40, Packard).

## 2.5 In Vivo Studies

### 2.5.1 Animal Maintenance

Han Wistar, dwarf (dw/dw) and the parent strain of the dwarf (AS) male and female rats were routinely used for the *in vivo* studies. All three strains were maintained under conditions of controlled lighting (lights on from 05.00 to 19.00h) and temperature (22°C) and water and food *ad libitum*. Following surgery all

animals were monitored closely, and any rat which appeared to be suffering or in ill health was killed.

### 2.5.2 Adrenal Steroid Manipulation Studies

#### 2.5.2.1 *Adrenalectomy ± Adrenal Steroid Replacement*

Male Han Wistar rats (200-250g; n=6/group) were anaesthetised with 4% halothane and bilaterally adrenalectomised through dorsal incisions. Control animals were sham-operated by making bilateral dorsal subcutaneous incisions under halothane anaesthesia. Adrenalectomised animals were injected subcutaneously with high dose dexamethasone (0.2mg/kg/day), a selective glucocorticoid which is poorly metabolised by 11ß-OHSD, aldosterone (20µg/kg/day), which is a selective MR agonist, or vehicle (5% ethanol saline). Sham-operated controls also received vehicle. Adrenalectomised rats were maintained on physiological saline to maintain electrolyte balance in these animals.

#### 2.5.2.2 *Arthritis Stress*

The model used to imitate chronic stress was achieved by inducing arthritis in male Han Wistar rats (200-250g; n=10/group) under 4% halothane anaesthesia. Animals were injected subdermally with Freund's Complete Adjuvant containing 250µg attenuated Mycobacterium tuberculosis (MAFF, UK) suspended in a total volume of 0.05ml paraffin oil, at two sites around the left tarsal joint (adjuvant injection was performed by Lucy Donaldson). Control animals were left completely untreated. Groups of animals were also adrenalectomised three days prior to induction of arthritis or treated with the glucocorticoid antagonist RU38486 (10mg/kg/day) by gavage or subcutaneous injection, while two further groups of rats were treated with vehicle (50% ethanol-saline by gavage or 10% DMSO by subcutaneous injection). Rats were killed 14 days after induction of arthritis or after 14d treatment with RU38486. During the development of arthritis all animals were monitored closely, but showed little or no apparent signs of discomfort and locomotor activity was nearly normal, with only occasional guarding of the affected limbs. Trunk blood samples were collected first thing in the morning to determine corticosteroid binding globulin and corticosterone levels in these animals. Thymus and adrenal weights were also measured as additional indicators of chronically raised corticosteroid levels.

### 2.5.3 Sex Steroid Manipulation Studies

#### 2.5.3.1 *11ß-OHSD and the Oestrus Cycle*

Vaginal smears were taken from female Han Wistar rats (150-200g) every day, and the cycling stage of each rat determined microscopically. Pro-oestrus is characterised by epithelial and cornified cells which tend to clump together. During oestrus only cornified cells are apparent, while the final stage, met-oestrus again exhibits cornified and epithelial cells, but in contrast to pro-oestrus the cornified cell walls begin to break down in this stage of the cycle. The animals were divided into groups of pro-oestrus, oestrus and met-oestrus (n=5/group), and were killed.

#### 2.5.3.2 *Gonadectomy ± Sex Steroid Replacement*

Groups of male Han Wistar rats (200-250g; n=5-6/group) and age-matched female Han Wistar rats (n=6/group) were gonadectomised or sham-operated under 4% halothane anaesthesia. Male animals were gonadectomised by a ventral incision through the scrotum followed by tying off the vas deferens. The testicles were then removed by cutting below the sutures. Female animals were ovariectomised by bilateral dorsal incisions and removal of both ovaries.

While still anaesthetised, gonadectomised animals were implanted subcutaneously with silastic capsules (1.98mm internal diameter, 12mm in length) containing testosterone propionate or 17ß-oestradiol. Sham-operated controls and a group of gonadectomised animals received blank capsules. Capsules were made by sealing one end of a length of medical grade silastic tubing with silastic sealant (both from Dow corning, Midland, Michigan). The sealant was allowed to set overnight before completely filling the capsules with the appropriate hormone and sealing the other end of the capsule. Before using, capsules were equilibrated for at least 4h in 0.9% NaCl. Rats were killed 48h or 10d after surgery.

#### 2.5.3.3 *11ß-OHSD and Pregnancy*

As a model for high physiological oestrogen levels, a group of female Han Wistar rats were impregnated, and on day 19 of pregnancy this group and a group of non-pregnant controls (n=6/group) were killed. Full term pregnancy in Han Wistar rats is 21-22 days.

### 2.5.4 Growth Hormone Manipulation Studies

#### 2.5.4.1 *Female Versus Male GH Patterns*

The normal male and female rats used in this study are from a colony inbred at the NIMR at Mill Hill in London called NIMR/AS. The dwarf male and female rats were originally derived from a spontaneous mutation in Lewis rats resulting in a growth hormone (GH) deficiency. However due to breeding problems in Lewis rats, the mutation was transferred to the NIMR/AS strain, and homozygous dwarfs mated with heterozygous partners until a pure colony of homozygous dwarf (dw/dw) rats was established. These dwarf rats appear to produce insufficient GH from the pituitary, while other hormone levels are unaffected (Charlton et al., 1988). Groups (n = 6-16/group) of male and female dwarf rats were age-matched with parent strain animals (AS). A group of dwarf male animals were implanted subcutaneously with osmotic mini-pumps (Charles River) containing human GH (200µg/day; Lilly) to mimic the normal female pattern of GH secretion. A group of dwarf female animals received 3 min intravenous pulses of human GH (25µg/3 min pulse) every 3h via an automatic injection system anchored to the skull plate to mimic the normal male pattern of GH secretion (Clark et al., 1985; carried out by T. Wells, NIMR, Mill Hill, London). Controls received vehicle. Rats were killed after 6d treatment.

#### 2.5.4.2 *Hypophysectomy ± Growth Hormone ± Oestradiol*

Groups (n=5/group) of male and female Wistar rats were hypophysectomised, and a group of male Wistar rats sham-operated under halothane anaesthesia (Charles River) and allowed to recover for 14d after surgery. A group of the hypophysectomised male animals was implanted subdermally with silastic capsules containing 17ß-oestradiol (1.98mm internal diameter, 12mm long), while a second group was implanted subcutaneously with osmotic mini-pumps containing human GH (200µg/day) and a third group received both treatments. Rats were killed after 6d treatment.

## 2.6 Tissue Culture Studies

### 2.6.1 Maintenance of Cells in Culture

CV-1 cells are originally derived from African Green Monkey kidney and now contain no steroid receptors (Giguere et al., 1986; Arriza et al., 1987) or 11ß-OHSD activity or mRNA (Low et al., unpublished observations). COS-7 cells are derived from CV-1 cells, but contain t-antigen (Gluzman, 1981).

These cells were maintained in culture in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 100 units/ml penicillin, 100µg/ml streptomycin and 200mM L-glutamine (all from Northumbria Biologicals Ltd or Gibco BRL) as well as 10% foetal bovine serum (CV-1), or 10% newborn bovine serum (COS-7). Cells were grown in vented 75cm$^2$ flasks (Costar UK Ltd) at 37°C in 5% $CO_2$.

Confluent cells were split with 1x trypsin/EDTA solution (Northumbria Biologicals Ltd) as follows: The medium in which the cells were growing was aspirated off and discarded into Chloros. Cells were then rinsed in 2ml trypsin/EDTA to remove residual serum which inactivates the trypsin/EDTA, the wash aspirated and discarded again into Chloros. Another 2ml of trypsin/EDTA was added to each flask, and the cells incubated either at room temperature or at 37°C for approximately 5 min or until cells detached under gentle agitation from the surface of the flask. Then cells were split back 1 in 4, and either added to a fresh 75cm$^2$ flask or discarded into Chloros if cells were only to be maintained. 18ml of fresh culture medium (described above) was added to each flask and the cells once again incubated at 37°C in 5% $CO_2$ until confluent. If required, the medium on the cells was replaced by fresh medium between cell splitting. Generally for transfections, four 75cm$^2$ flasks of CV-1 or COS cells were maintained and required for each experiment.

### 2.6.2 11ß-OHSD Activity in Intact Cells Versus Cell Homogenates

In these experiments, the ability of 0.1-10µg pSL1 to act as a dehydrogenase or reductase in intact cells was examined over a 24h period. Then the dehydrogenase activity of 11ß-OHSD in homogenates of the same cells was measured as an indication of potential activity of the plasmid.

CV-1 cells were initially transfected using several methods, including electroporation, lipofection, DEAE dextran and calcium phosphate, but were found to transfect most efficiently with the calcium phosphate method of transfection (Low et al., unpublished observations). In contrast, COS-7 cells were routinely transfected by the DEAE dextran method.

Cells were harvested in 2ml trypsin/EDTA per 75cm$^2$ flask (section 2.6.1) and put in a 50ml falcon centrifuge tube (Costar UK) with some medium to inactivate the trypsin/EDTA solution. The cells were recovered by centrifugation at 3000rpm for 2 min in a Fisons MSE Chilspin centrifuge. The medium was aspirated off into Chloros, and the cell pellet resuspended in 10ml transfection medium. Cells were then counted using a haemocytometer, and 5x $10^5$ cells plated onto 100mm cell culture dishes in 10ml transfection medium which consists of DMEM containing 100 units/ml penicillin, 100µg/ml streptomycin and 200mM glutamine as well as 10%

charcoal stripped foetal calf serum (CV-1) or either 10% Nu serum or 10% foetal calf serum (COS-7). Charcoal stripping (section 2.6.2.4) was used to remove endogenous glucocorticoids from the serum. Nu-serum is a synthetic serum consisting of 25% foetal calf serum with various growth factors and steroids. The advantage of Nu-serum in the DEAE dextran method of transfection is that protein levels are very low, and high protein concentrations reduce the efficiency of transfection by this method. Unfortunately, Nu-serum contains 28µM cortisol (information provided Universal Biologicals, London, UK) which is probably not completely removed by charcoal stripping (charcoal stripping procedure removes 99.8% of added [$^3$H] corticosterone [section 2.6.2.4]. Thus of 28µM cortisol, approximately 56nM may still be left in the serum). These transfections were therefore also repeated in 10% foetal calf serum. Cells were plated on 100mm cell culture dishes (Costar UK) since insufficient protein was produced from smaller plates to allow the 11ß-OHSD assay to be performed at an appropriate protein concentration.

2.6.2.1 *DEAE Dextran Transfection of COS-7 Cells*

24h after plating, 0.1-10µg of SL1 or pJ3 in addition to 1-5µg pCH110 or pKC 275 (to control for transfection efficiency) was made up to 15µg with pGEM3 and mixed with DEAE dextran (final concentration= 250µg/ml) and chloroquine (final concentration=80µM) (Table 2.2). This DNA mixture was then added to the culture dishes dropwise while swirling.

It was found that DMSO shocking the cells 4-8h after adding the DNA to the cells increased the efficiency of transfection. The medium containing the DNA/DEAE dextran was aspirated off into Chloros, and replaced by 2ml 10% DMSO in 1x PBS for 2 min exactly. The DMSO was then aspirated off and replaced as quickly as possible by two volumes of serum free DMEM to wash the cells. This was repeated once more before the cells were returned to regular transfection medium and replaced in the incubator at 37°C and 5% $CO_2$.

To measure 11ß-dehydrogenase and 11ß-reductase activities in the intact cells, 24h after transfection, the medium was aspirated off the cells and replaced by 12ml of fresh growth medium containing 25nM corticosterone (23nM unlabelled B and 0.16µl [$^3$H]-B; specific activity 84Ci/mmol) or 25nM 11-dehydrocorticosterone (23nM unlabelled A and 0.16µl [$^3$H]-A; synthesised as described in section 2.6.2.5). Aliquots of 750µl medium were then removed from the culture dishes at various time points and steroids extracted with 1ml ethyl acetate, dried down, and resuspended in 100µl ethanol containing 2.5mg/ml each of unlabelled B and A, and the conversion of [$^3$H]-B to [$^3$H]-A or [$^3$H]-A to [$^3$H]-B determined by thin layer chromatography in the same way as for other 11ß-OHSD assays (section 2.4.1).

| | 60mm cell culture dishes | 100mm cell culture dishes |
|---|---|---|
| Volume of medium per plate | 4ml | 10ml |
| No. of cells plated/dish | $3x\ 10^5$ | $5x\ 10^5$ |
| Total amount of DNA | 10µg | 15µg |
| 10mg/ml DEAE dextran | 150µl | 250µl |
| 8mM Chloroquine | 40µl | 100µl |
| 10% DMSO | 1ml | 2ml |
| Harvesting buffer | 500µl TM triton | 1ml 1x PBS |

**Table 2.2:**
Requirements for DEAE Dextran Transfections

48h after transfection, the medium was aspirated from the plates into Decon, and the cells washed in 3ml 1xPBS. This was also aspirated off, and replaced with 1ml of 1x PBS. Cells were scraped into eppendorf tubes, and spun down at 14,000xg in a bench top eppendorf centrifuge for 2 min. The 1x PBS solution was aspirated off, and replaced by 100µl of TM triton (TM triton=0.1% triton X100 in 20mM Tris-HCl (pH 7.5), 2mM $MgCl_2$). From this suspension of cells, ß-galactosidase assays (section 2.6.5), protein assays, and 11ß-OHSD activity assays (section 2.6.2.3) were routinely performed.

2.6.2.2 *Calcium Phosphate Transfection of CV-1 Cells*

CV-1 cells were plated the day before the transfection experiment in the same way as COS-7 cells. For each transfection, 500µl of 2x HEPES buffered saline (HBS; 2x HBS= 50mM HEPES (pH 7.1), 280mM NaCl, 1.5mM $Na_2HPO_4$) was pipetted into sterile eppendorf tubes. In separate tubes, the DNA required for the transfection (0.1-10µg pSL1/pJ3, 1-5µg pCH110/pKC 275) made up to 15µg with pGEM3 was mixed with 62µl of 2M $CaCl_2$ and filter sterilised $mH_2O$ (millipore $H_2O$) to a volume of 500µl. While vortexing the 2x HBS, the prepared DNA solution was added dropwise so that the resulting solution appeared slightly opaque due to the formation of a fine co-precipitate of DNA with calcium phosphate. It was found that

transfections were most efficient if the 2x HBS and $CaCl_2$/DNA solutions were mixed at 20-21°C. The DNA/$CaPO_4$ solution was incubated at room temperature for 30 min, then vortexed again just prior to adding it to the cells. The DNA was added dropwise to the plates while swirling to evenly distribute the precipitate, and to avoid local acidification of the cells. The plates were then returned to the incubator at 37°C and 5% $CO_2$. 4-8h later, cells were shocked for exactly 2 min with 15% glycerol (30% glycerol diluted down to 15% with 2x HBS prior to shocking), but were otherwise treated in the same way as COS-7 cells.

2.6.2.3 *In Vitro 11ß-OHSD Enzyme Assay in Cell Extracts*

48 hours after transfection the cells were harvested in 1x PBS, the cells spun down, and the pellet resuspended in TM triton. After estimating the total protein concentration of each cell suspension colorimetrically (Bio-Rad protein assay kit), 500µg/ml aliquots of cell homogenates were incubated with 1mM NADP and 60nM 1,2,6,7 [$^3$H] B in a total volume of 20µl with Krebs Ringer buffer supplemented with 0.2% glucose and 0.2% BSA, for 10 min at 37°C. Steroids were extracted with at least 2 volumes ethyl acetate and the conversion of [$^3$H]-B to [$^3$H]-A was estimated by thin layer chromatography in the same way as for intact cells.

2.6.2.4 *Charcoal Stripping of Serum*

0.25% charcoal and 0.0025% dextran T70 were incubated overnight at 4°C in 0.25M sucrose, 1.5mM $MgCl_2$ and 10mM Hepes (pH 7.4) on a clock stirrer so the suspension was completely mixed. 50ml of the charcoal suspension was then centrifuged at 3000rpm for 10 min. to pellet the charcoal. The supernatant was discarded and replaced with an equivalent volume of the serum to be stripped. This mixture was vortexed thoroughly, and incubated at 4°C overnight on the clock stirrer again, before being filtered through a 0.22µm bottle filter (Costar, UK) into a 500ml bottle of DMEM. To check the efficiency of the stripping procedure, 1µl [$^3$H]-B was added to an aliquot of serum to be stripped. The amount of radioactivity present in the serum was then checked before and after the stripping procedure on a ß-counter. The stripping procedure removed >99.8% [$^3$H]-B.

2.6.2.5 *Synthesis of [$^3$H] 11-Dehydrocorticosterone*

[$^3$H] 11-dehydrocorticosterone was made using human placental tissue extract in a modification of the method reported by Lakshmi and Monder (1988). Human placenta is a pure source of 11ß-dehydrogenase activity (Brown et al., 1993a) and was therefore found to be the best tissue producing the least contamination with corticosterone.

Routinely, placental extract was incubated with 1mM NAD in 0.1M Tris-HCl (pH 7.5) buffer containing 12nM [$^3$H]-B in a total volume of 5ml, at 37°C for at least 2h. The steroids were then extracted with 2 volumes of ethyl acetate twice, and dried down under air. A sample of the steroids was dried down and then resuspended in 600µl of 50:50 methanol: HPLC $H_2O$ and run through an HPLC to check the purity of the [$^3$H]-A. Using this method, purity was at least 98% [$^3$H]-A. The [$^3$H]-A was resuspended in an appropriate volume of ethanol to give approximately the same specific activity as the [$^3$H]-B used in the transfection experiments. [$^3$H]-A was stored at -20°C for up to months, but was checked again on the HPLC before using because it has a tendency to reform [$^3$H]-B.

### 2.6.3 Effect of 11ß-OHSD on MMTV-LTR Luciferase Activity

For this assay, COS-7 cells were plated on 60mm dishes in 4ml growth medium containing the same additives as previously described (section 2.6.2.2). This assay was carried out to determine the ability of human glucocorticoid receptor (hGR) to activate a glucocorticoid responsive reporter gene (MMTV-LTR luciferase) in the presence or absence of various concentrations of unlabelled B or A. In each transfection assay, there was 1µg pKC275 as an internal control, 1µg hGR, 5µg MMTV-LTR luciferase or RSV-luciferase, and 10µg of pSL1 or pJ3.

Transfection was carried out in exactly the same way as described in section 2.6.2.1, except in smaller quantities (Table 2.2). After DMSO shocking the cells, fresh transfection medium was supplemented with 0.1nM-1µM unlabelled B or A or 10nM dexamethasone or no steroid supplement at all. 48h after transfection, cells were washed in 1ml of 1x PBS and harvested into eppendorf tubes with 500µl lysis buffer in which luciferase assays and ß-galactosidase assays could be performed. The lysis buffer consisted of 25mM Tris-phosphate (pH 7.8), 8mM $MgCl_2$, 1mM DTT, 1mM EDTA, 1% triton X100, 1% BSA and 15% glycerol. This buffer was made up fresh on the day of harvesting.

### 2.6.4 Efficiency of Transfection

COS-7 cells were plated in 100mm$^2$ cell culture dishes and transfected with 15µg pCH110 or pKC 275 as described in section 2.6.2.2. 48h following transfection, cells were fixed in 0.5% glutaraldehyde in 1x PBS for 15 min at room temperature. After washing in 1x PBS, the cells were incubated in 3.3mM $Fe_4K(CN)_6.3H_2O$, 3.3mM $Fe_3K(CN)_6$, 1mM $MgCl_2$, and 150mM NaCl in 10mM sodium phosphate buffer (pH7.0) containing 0.1% X-Gal (5-bromo-4-chloro-indolyl-b-D-galactosidase) for 2h

at 37°C. The reaction was stopped by replacing the X-Gal solution with 70% glycerol. The percentage of transfected cells was determined microscopically. As a control, COS-7 cells were transfected with pGEM3 and incubated in 0.1% X-Gal in exactly the same way.

### 2.6.5 ß-Galactosidase Assay

Plasmids encoding ß-galactosidase were routinely incorporated into transfection experiments to determine the transfection efficiency.

For each sample to be assayed, 50µl of cell extract was mixed with 181µl 0.1M sodium phosphate; pH 7.5 (41ml 0.2M $Na_2HPO_4.2H_2O$, 9ml 0.2M $NaH_2PO_4.2H_2O$, 50ml $dH_2O$), 66µl 1x ONPG (o-nitrophenyl-ß-D-galactopyranoside; 1x ONPG= 4mg/ml ONPG in 0.1M sodium phosphate) and 3µl 100x Mg solution (0.1M $MgCl_2$, 4.5M ß-mercaptoethanol). This reaction was incubated at 37°C until a yellow colour developed. The reaction was stopped by adding 500µl 1M $Na_2CO_3$ to each reaction. The optical density of the reactions was measured at a wavelength of 420nm. From these absorbance readings, each transfection result could be corrected depending on the transfection efficiency.

### 2.6.6 Luciferase Assay

The firefly enzyme luciferase catalyses the ATP-dependent oxidation of luciferin. For every mole of luciferin oxidised, one photon is released which can then be detected on a scintillation counter, or in this case, the more accurate luminometer.

The reaction mix for luciferase assays was made up in 500µl eppendorf tubes. The reaction mix consisted of 50µl cell extract, 200µl lysis buffer (25mM Tris-phosphate (pH 7.8), 8mM $MgCl_2$, 1mM DTT, 1mM EDTA, 1% triton x-100, 15% glycerol and 1% BSA) and 4µl 20mM ATP, and placed in the counting chamber of a Berthold luminometer. 100µl of 0.25 mM luciferin was injected into the counting chamber and an instantaneous measurement of light produced was recorded.

## 2.7 Statistics

Adrenal steroid, sex steroid and growth hormone manipulation studies were analysed by Analysis of Variance followed by Duncan's Multiple Range Test. Significance was set at $p<0.05$. Values are expressed as means±SEM. Transfection studies were not statistically analysed.

# CHAPTER 3

# GLUCOCORTICOID REGULATION OF 11ß-OHSD

---

## 3.1 Introduction

The liver is a major target organ for the actions of glucocorticoids. For example, glucocorticoids maintain glycogen reserves in the liver by activating glycogen synthase and inactivating glycogen phosphorylase. Glucocorticoids may also have effects on fatty acid metabolism and insulin control. In addition, liver is the organ which expresses the highest levels of 11ß-OHSD activity (Monder & Shackleton, 1984). However, it is widely believed that the predominant direction of 11ß-OHSD in liver is reductase (Bush, 1969) and therefore hepatic 11ß-OHSD will presumably have a very different role to the proposed actions in aldosterone-sensitive tissues. Glucocorticoids also have multiple effects on the brain in the regulation of mood and behaviour as demonstrated by the raised frequency of psychological disturbances (eg depression) being found in clinical syndromes of glucocorticoid excess, such as Cushing's syndrome (Cohen, 1980). The hippocampus in particular is extremely vulnerable to glucocorticoids, such that chronic glucocorticoid exposure during stress or ageing for example, especially in combination with other chronic insults such as ischaemia, can result in the specific degeneration of hippocampal neurones in the CA1 and CA3 fields (Sapolsky et al., 1985; Sapolsky & Pulsinelli, 1985; Sapolsky et al., 1986). Glucocorticoid deficiency also results in specific hippocampal degeneration (Sloviter et al., 1989). Therefore a mechanism must exist to regulate glucocorticoid levels in hippocampal neurones, and presumably other regions of the brain. 11ß-OHSD activity (Lakshmi et al., 1991), mRNA expression (Moisan et al., 1990a) and immunohistochemistry (Sakai et al., 1992) have been demonstrated in various regions of the brain, with high levels expressed in hippocampus. It has therefore been suggested that 11ß-OHSD may be the selection mechanism responsible for regulating glucocorticoid access to hippocampal neurones *in vivo*. In support of this, 11ß-OHSD activity in hippocampus during development falls from postnatal day 1 to a nadir at postnatal day 10, only to rise again in adult rats, thus following glucocorticoid levels during development (Moisan et al., 1992a). In addition, sequencing of the 'liver-type' 11ß-OHSD gene promoter revealed potential

glucocorticoid response elements indicating a possible direct interaction between glucocorticoids and the 11ß-OHSD gene (Moisan et al., 1992b). 11ß-OHSD may also be appropriately regulated by long term exposure to chronically raised glucocorticoid levels found for example in long term stress. Indeed, regulation of 11ß-OHSD activity by glucocorticoids has been demonstrated in cultured normal human foreskin fibroblasts, where 11ß-OHSD activity was increased by the addition of dexamethasone, cortisol and corticosterone to the cell culture medium (Hammami & Siiteri, 1991). In contrast, *in vivo* glucocorticoid manipulations had no effect on rat kidney 11ß-OHSD activity (Smith & Funder, 1991). I have begun to address the possibility of 11ß-OHSD regulating glucocorticoid access to hippocampal neurones by firstly demonstrating the co-localisation of 'liver-type' 11ß-OHSD mRNA with both MR and GR mRNAs, and furthermore investigating whether 11ß-OHSD is regulated by adrenal steroid manipulations. I have also considered regulation of 11ß-OHSD using a model of chronic stress, the arthritic rat, to mimic physiologically raised levels of glucocorticoids.

## 3.2 Results

### 3.2.1 Co-Localisation of 11ß-OHSD with MR and GR in Hippocampus

For 11ß-OHSD to be involved in regulating access of glucocorticoids to mineralocorticoid and glucocorticoid receptors, it is likely that the enzyme would be co-localised in cells containing these receptors. Therefore *in situ* hybridisation was used to investigate whether 11ß-OHSD mRNA is co-localised with MR and GR mRNAs in hippocampus.

Firstly, non-isotopic *in situ* hybridisation in hippocampus was developed using biotin-labelled MR, GR and 11ß-OHSD cRNA probes. These probes were hybridised to 10µm coronal hippocampal tissue sections and developed using an avidin-biotin-horseradish peroxidase complex with 3, 3'-diaminobenzidine (DAB) as substrate for the enzyme. Figure 3.1 shows DAB stained 11ß-OHSD mRNA in hippocampus in comparison to tissue sections hybridised under the same conditions in the absence of probe. As previously described for $^{35}$S-labelled 11ß-OHSD cRNA probes (Moisan et al., 1990a), biotin-labelled 11ß-OHSD cRNA probes demonstrated the presence of 11ß-OHSD mRNA in all regions of the hippocampus and dentate gyrus with highest expression found in the CA3 subfield. In the same way, optimal conditions for hybridisation of biotin-labelled GR cRNA probes were determined. GR mRNA was highly expressed in dentate gyrus, CA1 and CA2 but showed lower expression in CA3 and CA4 subfields (data not shown) as previously described for

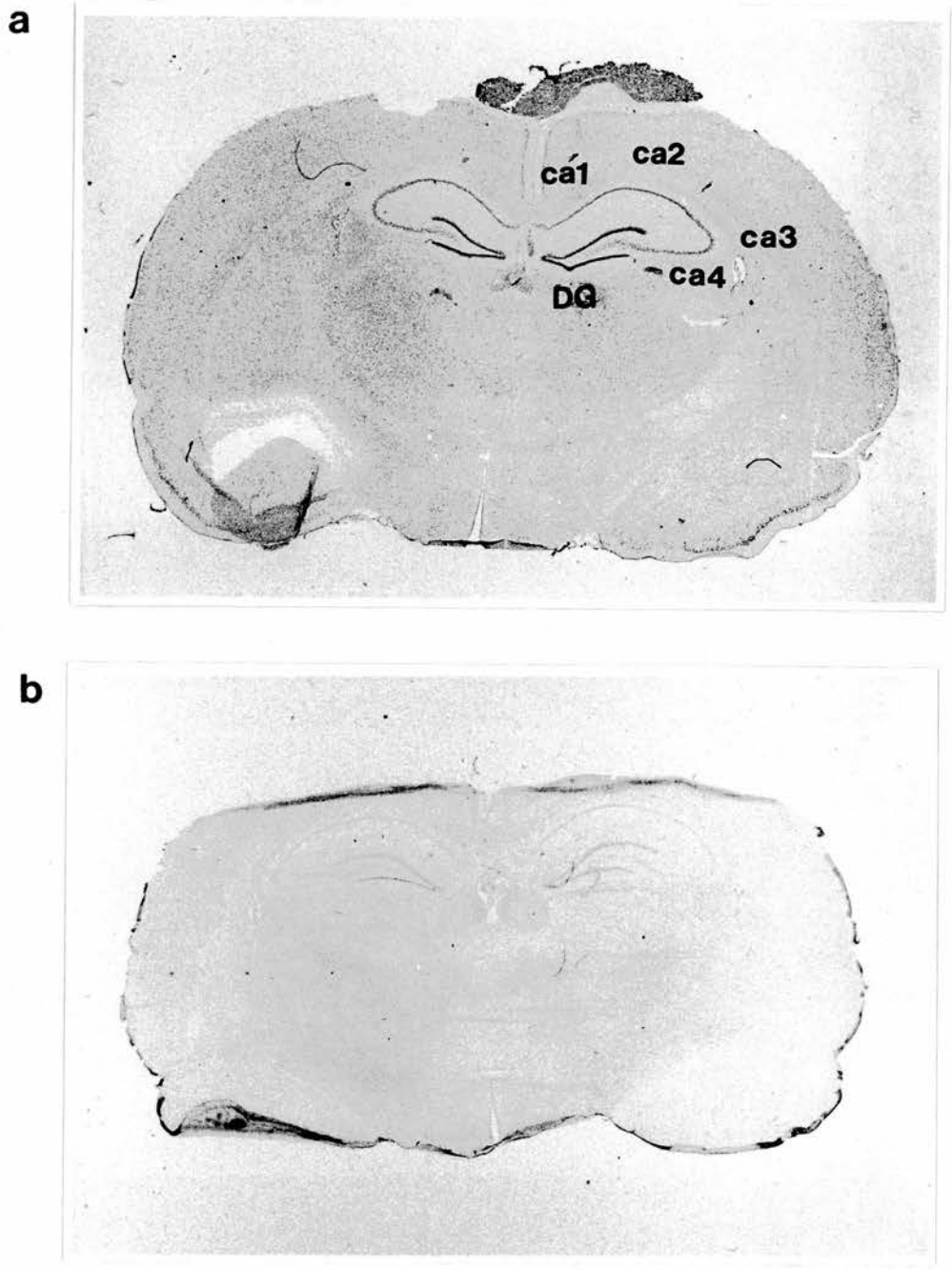



**Figure 3.1:**

*In situ* hybridisation of 11ß-OHSD mRNA in rat hippocampus using (a) a biotin-labelled 11ß-OHSD cRNA probe, and (b) no probe. Biotin staining was visualised using an avidin-biotin-horseradish peroxidase complex with 3,3-diaminobenzidine as substrate.

$^{35}$S-labelled GR probes (Arriza et al., 1988; Van Eekelen et al., 1989; Sousa et al., 1989). For both of these probes, competition studies were also performed to determine the specificity of hybridisation. Hybridisation of biotin-labelled 11ß-OHSD was attenuated by competition with 100-fold excess unlabelled 11ß-OHSD cRNA probe, but not by excess unlabelled GR probe. In the same way, hybridisation of biotin-labelled GR probes was decreased by homologous but not heterologous probe (data not shown). Unlike biotin-labelled 11ß-OHSD and GR probes, optimal conditions for specific hybridisation of biotin-labelled MR probes could not be determined.

Double *in situ* hybridisation was performed by simultaneously hybridising $^{35}$S-labelled MR or GR cRNA probes with a biotin-labelled 11ß-OHSD cRNA probe. Following hybridisation and stringent washing, biotin-labelled 11ß-OHSD was again detected using DAB as substrate for the horseradish peroxidase detection system. Slides were then put against autoradiographic film and/or dipped in emulsion to localise the $^{35}$S-labelled MR and GR mRNAs. 11ß-OHSD was again found in all regions of the hippocampus (Fig. 3.2a). Similarly, autoradiographs of MR in hippocampus showed characteristic binding in all regions of the hippocampus indicating probable co-localisation with 11ß-OHSD (Fig. 3.2b). However following exposure to nuclear emulsion, it was found that DAB is silver enhanced, and therefore is not a useful substrate for co-localisation studies for *in situ* hybridisation.

Co-localisation of 11ß-OHSD with both MR and GR was confirmed using AEC (which is not silver enhanced) as substrate for the horseradish peroxidase detection system. At a microscopic level, 11ß-OHSD mRNA was found in the pyramidal and granule cell neuronal layers of the hippocampus with highest expression in the CA3 subfield in agreement with previous findings (Moisan et al., 1990a). MR (Fig. 3.3a) and GR (Fig. 3.3b) mRNAs as indicated by the presence of silver grains, are both found in regions of the hippocampus containing 11ß-OHSD mRNA, thus demonstrating for the first time that 11ß-OHSD and corticosteroid receptors are colocalised in rat hippocampus.

### 3.2.2 Effects of 10d Adrenal Manipulations on 11ß-OHSD

To examine the effects of adrenal steroids, groups of rats were adrenalectomised and then replaced either with high dose dexamethasone (200µg/kg/day), a selective GR agonist which is poorly metabolised by 11ß-OHSD, or aldosterone (20µg/kg/day), which is a selective MR agonist. After 10d treatment, 11ß-OHSD activity and mRNA levels were measured.

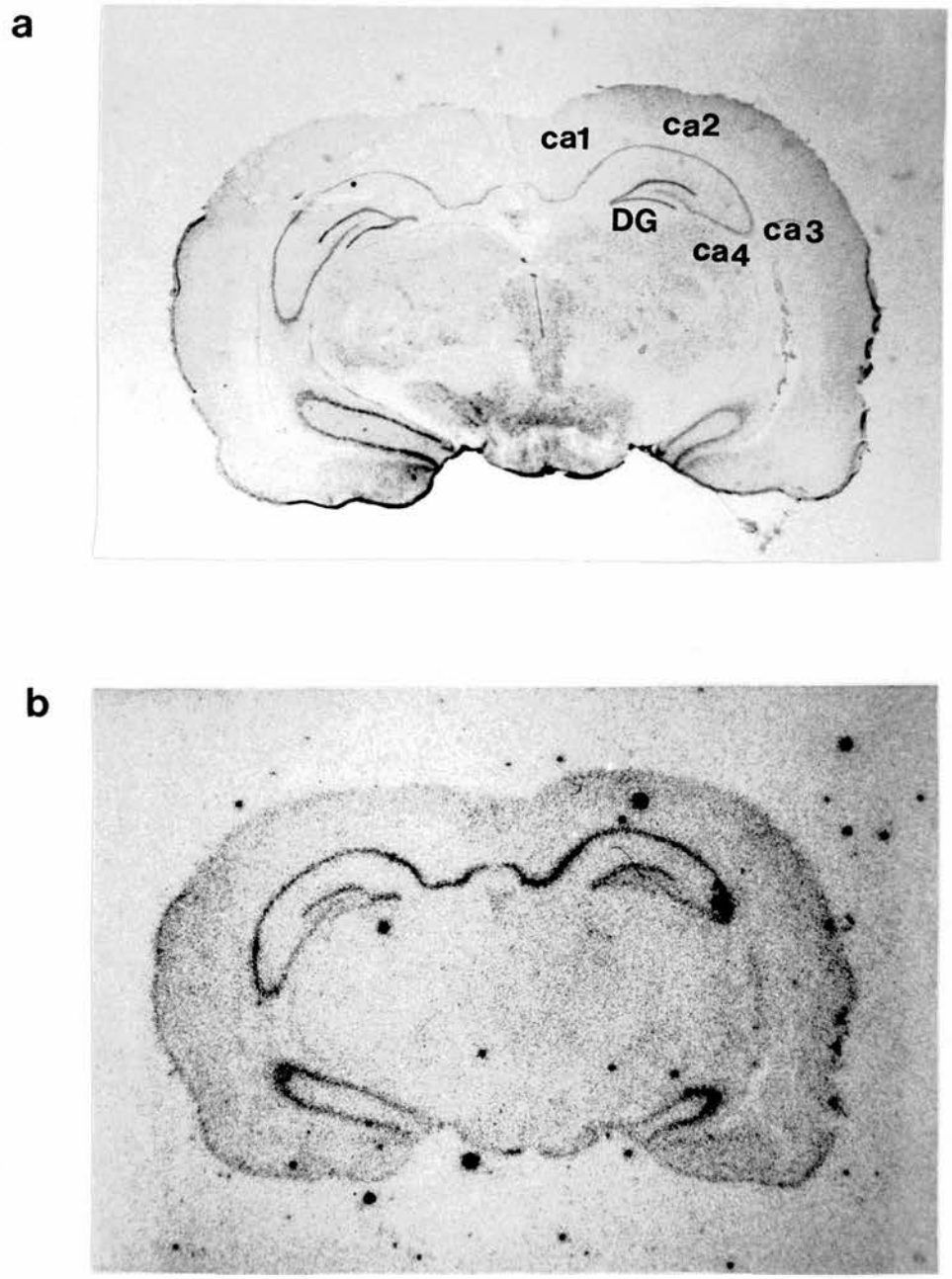



**Figure 3.2:**

Co-localisation of 11ß-OHSD mRNA with MR mRNA. Hippocampal tissue sections were hybridised simultaneously with a biotin-labelled 11ß-OHSD cRNA probe and a $^{35}$S-labelled MR cRNA probe. (a) 11ß-OHSD mRNA visualised with 3,3-diaminobenzidine. The same hippocampal sections were then exposed to autoradiographic film to determine the hippocampal localisation of (b) MR mRNA.

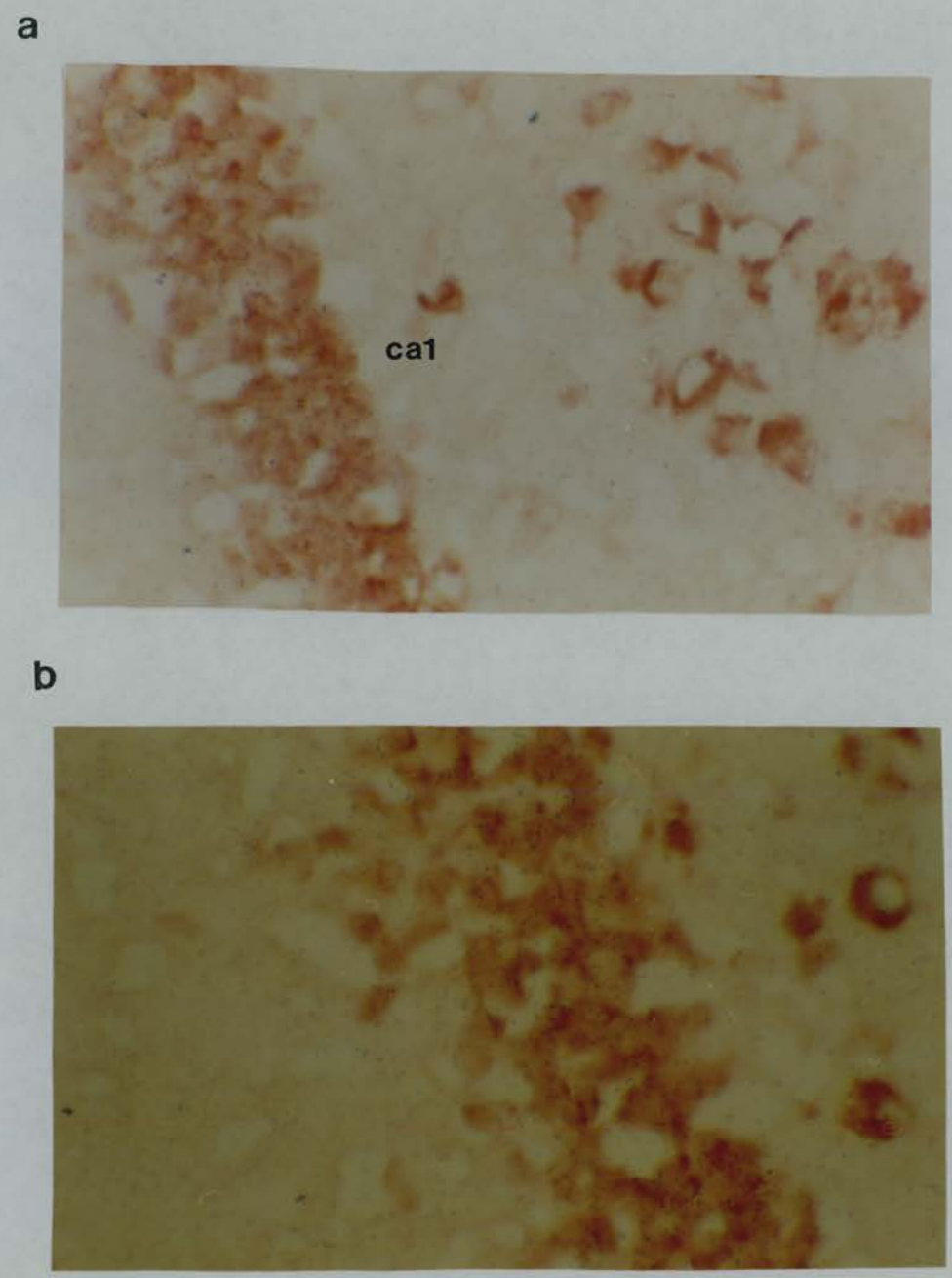



**Figure 3.3:**

Co-localisation of 11ß-OHSD mRNA with MR and GR mRNAs. Hippocampal tissue sections were hybridised simultaneously with biotin-labelled 11ß-OHSD cRNA probes and $^{35}S$-labelled MR or GR cRNA probes. Biotin label was detected using 3-amino-9-ethylcarbazole as the substrate. The same hippocampal tissue sections were then dipped in nuclear emulsion for 28d. (a) Co-localisation of 11ß-OHSD mRNA with MR mRNA and (b) GR mRNA in the CA1 subfield of the hippocampus. Co-localisation is demonstrated by the presence of silver grains over cells stained for the presence of the biotin label. Photographs were taken at 40x magnification.

Adrenalectomy led to a decrease in 11ß-OHSD activity in liver, but in contrast had no effect on hippocampal 11ß-OHSD activity (Fig. 3.4a). Dexamethasone, but not aldosterone, treatment reversed the decrease in hepatic 11ß-OHSD activity to control levels, and significantly increased hippocampal 11ß-OHSD activity (23% rise compared to adrenalectomy) (Fig. 3.4a). None of the adrenal steroid manipulations had any effect on 11ß-OHSD activity in kidney (Fig. 3.4a). It is interesting to note that 11ß-OHSD activity in sham-operated controls was low in hippocampus (similar to levels expressed in adrenalectomised animals) while 11ß-OHSD activity was high (similar to chronic dexamethasone treatment) in liver (Fig. 3.4a).

To determine whether changes in 11ß-OHSD activity in hippocampus and liver occurred at the level of transcription, the effects of adrenalectomy alone or in combination with dexamethasone replacement were also examined by northern analysis. In hippocampus, adrenalectomy led to a decrease in 'liver-type' 11ß-OHSD mRNA expression (32% decrease compared to sham-operated controls), while dexamethasone treatment increased 11ß-OHSD mRNA expression by 69% when compared to adrenalectomy alone (Figs. 3.4b, 3.5). In a similar way, 10d dexamethasone treatment resulted in a non-significant rise in hepatic 11ß-OHSD mRNA expression (48% increase), while mRNA levels in sham-operated controls were similar to those found in glucocorticoid treated animals (Fig. 3.4b, 3.5). Adrenalectomy alone or followed by dexamethasone replacement had no effect on any of the transcripts of 'liver-type' 11ß-OHSD which hybridise to the known 11ß-OHSD cDNA in kidney (Fig. 3.4b, 3.5).

### 3.2.3 Arthritic Stress and 11ß-OHSD

In order to determine whether a physiological stress has similar regulatory effects on 11ß-OHSD as dexamethasone, a model of adjuvant-induced arthritis was used. Similar models of arthritis in rats have been shown to chronically activate the hypothalamic-pituitary-adrenal axis (Sarlis et al., 1992). Corticosterone and CBG were measured in arthritic animals to assess the effects of long term stress, as were adrenal weight and thymus weight. 14d after injection of 250µg attenuated Mycobacterium tuberculosis around the left tarsal joint, chronic arthritis was apparent (monitored by Lucy Donaldson). At this time, adrenal weight was significantly raised and thymus weight significantly decreased compared to untreated controls. In addition, morning plasma corticosterone levels were raised and CBG levels were reduced compared to controls, although these changes did not reach statistical significance (Table 3.1). These differences are indicative of chronic glucocorticoid hypersecretion and suggested that these animals have been subjected to chronic

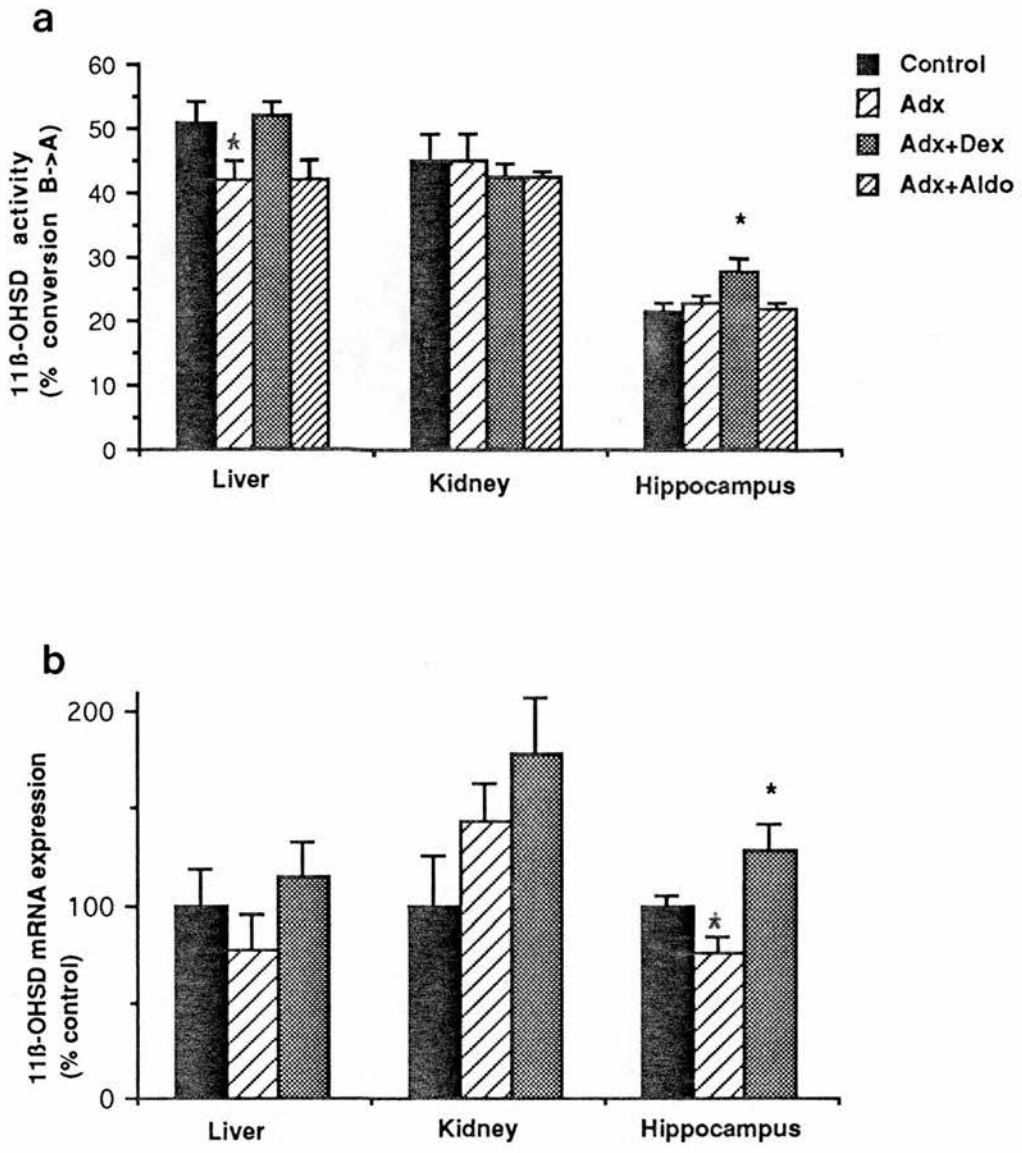



**Figure 3.4:**

The effect of adrenalectomy alone (Adx) or followed by dexamethasone treatment (Adx+Dex) or aldosterone treatment (Adx+Aldo) for 10d on (a) 11ß-OHSD activity or (b) 'liver-type' 11ß-OHSD mRNA levels in male rat liver, kidney and hippocampus. 11ß-OHSD activity is expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A. 'Liver-type' 11ß-OHSD mRNA levels are expressed as a percentage of sham-operated controls. $^*p<0.05$ compared to sham-operated controls (Duncan's Multiple Range test). n=6/group.

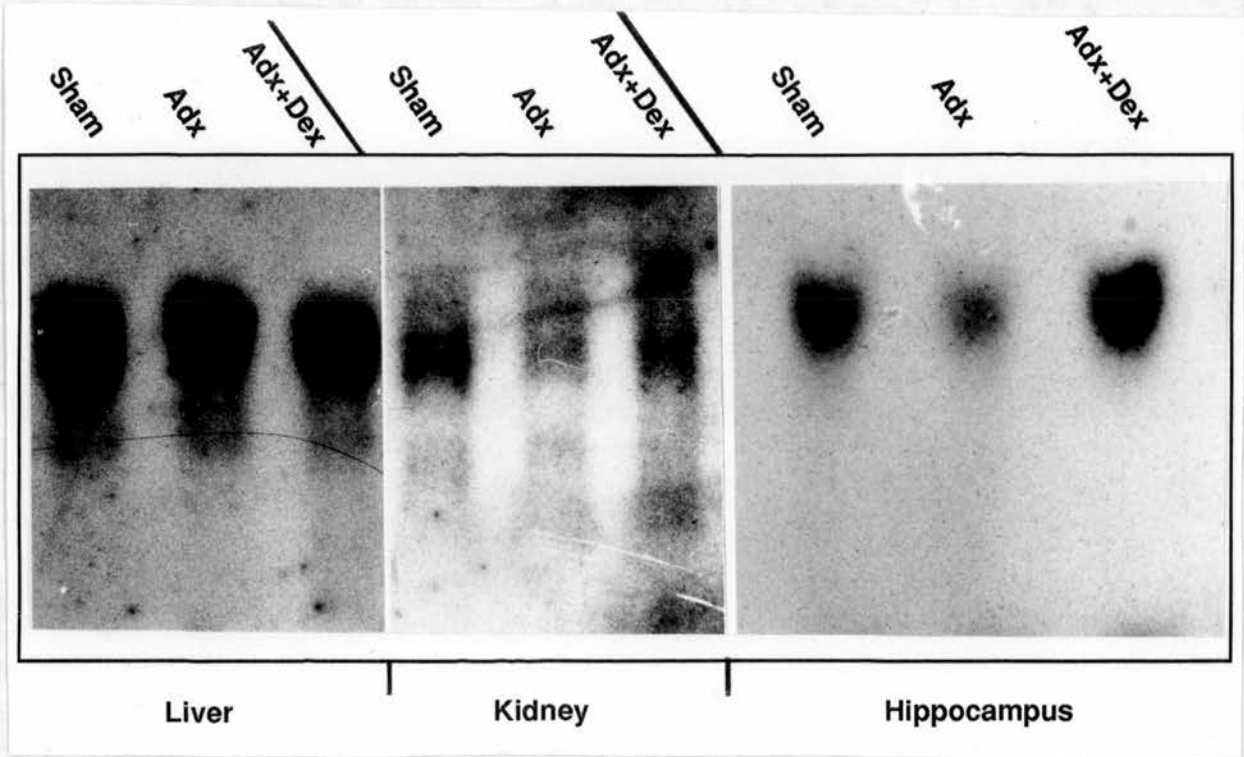



**Figure 3.5:**

Northern blot analysis of 11ß-OHSD mRNA hybridised with $^{32}$P-labelled 'liver-type' 11ß-OHSD cDNA showing the effects of adrenalectomy (Adx) alone or followed by dexamethasone treatment (Adx+Dex) compared to sham-operated controls (Sham) in male rat liver, kidney and hippocampus. Each lane represents RNA extracted from a single individual. 11ß-OHSD mRNA expression was quantified by computer densitometry and expressed as a ratio of the optical densities of 11ß-OHSD:7S. The 11ß-OHSD mRNA species in liver and hippocampus is 1.7 kb, while in kidney there are multiple mRNA species of 1.5, 1.6, 1.7 and 1.9 kb.

| | Corticosterone (nmol/l) | CBG (pmol/mg) | Thymus weight (g) | Adrenal weight (g) |
|---|---|---|---|---|
| Controls | 337±35 | 13.2±1.9 | 0.66±0.08 | 0.07±0.01 |
| Arthritis | 490±91 | 7.5±2.1 | 0.46±0.04* | 0.10±0.01* |
| Adx+Arthritis | ND | 24.6±7.9 | 0.69±0.08 | ----- |

**Table 3.1:**
Plasma corticosterone and CBG levels and thymus and adrenal weights in completely untreated controls, arthritic rats 14 days after mycobacterium tuberculosis injection around the left tarsal joint, and similarly arthritic rats which had been adrenalectomised 3 days prior to the induction of arthritis. n=5/group. ND= below the limits of assay detection. *$p<0.05$ compared to control values (Duncan's Multiple Range tests).

stress. The effects of arthritis on hippocampal 11ß-OHSD were in agreement with the effects of dexamethasone treatment; hippocampal 11ß-OHSD activity was significantly increased in arthritic animals when compared to controls (20% increase) (Fig. 3.6a). Adrenalectomy 3d prior to induction of arthritis prevented the increase in hippocampal 11ß-OHSD activity, although the degree of arthritis (estimated by measuring joint circumference) was similar in both groups (measurements made by Lucy Donaldson). In contrast, adrenalectomy led to a non-significant decrease in hippocampal 11ß-OHSD mRNA expression (41% decrease), while induction of arthritis had no effect compared to untreated controls (Fig. 3.6a). Similarly in liver, induction of arthritis had no effect on 11ß-OHSD mRNA expression, whereas prior adrenalectomy led to a decrease in 11ß-OHSD mRNA expression of 56% (Fig. 3.6b). However, hepatic 11ß-OHSD activity was not affected in this model of stress (Fig. 3.6a).

### 3.2.4 Effects of RU38486 on 11ß-OHSD

The experiments described in section 3.2.3 suggested that in arthritic rats the arthritis-induced rise in 11ß-OHSD activity was glucocorticoid mediated. To

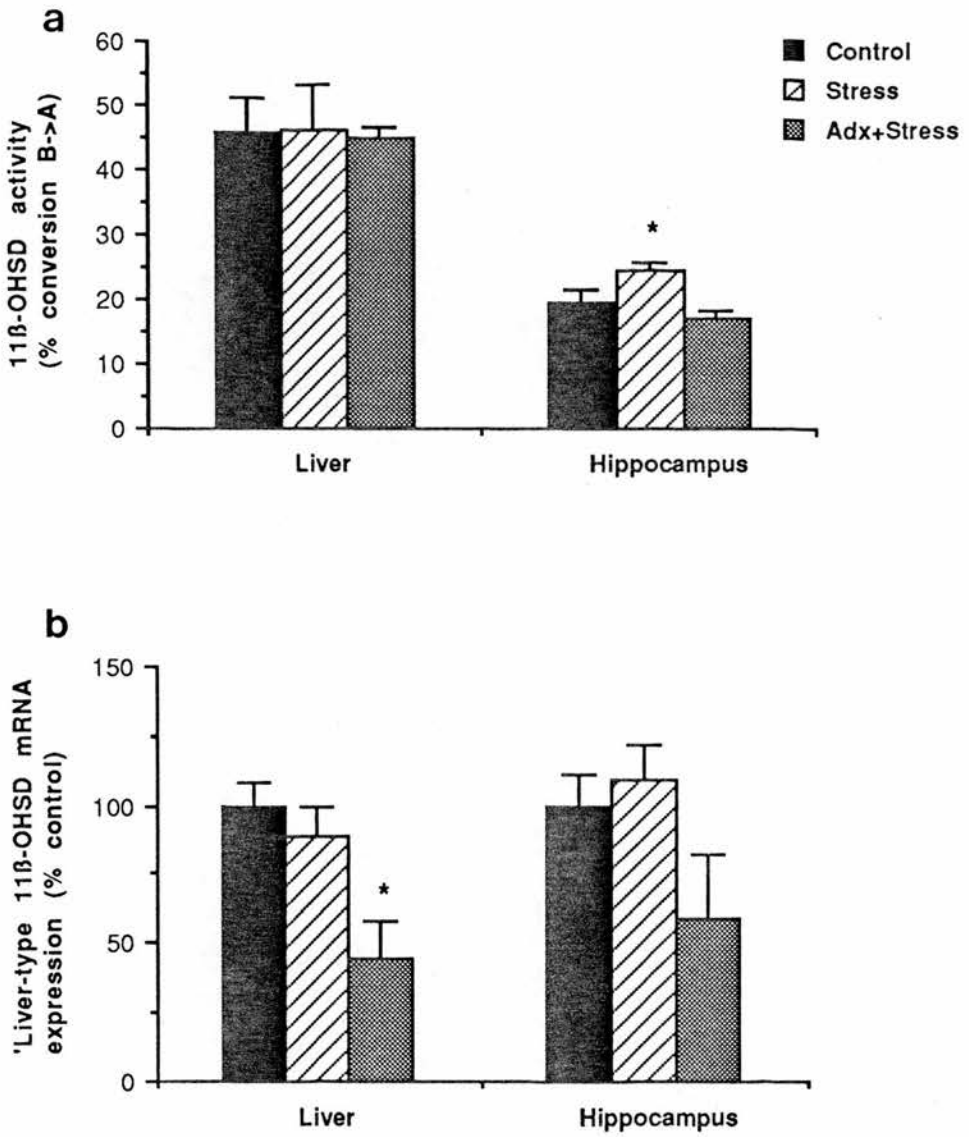



**Figure 3.6:**

Effect of 14d arthritis (stress) and arthritis with prior adrenalectomy (Adx+stress) compared to untreated control rat liver and hippocampus. (a) 11ß-OHSD activity expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A and (b) 'liver-type' 11ß-OHSD mRNA levels expressed as a percentage of untreated controls. $^*p<0.05$ compared to untreated controls (Duncan's Multiple Range test). n=10/group.

substantiate this suggestion, 11ß-OHSD activity was also measured in intact arthritic rats treated for 14d with the glucocorticoid antagonist RU38486 (10mg/kg/day) either in 10% DMSO by subcutaneous injection, or in 50% ethanol by gavage in two separate experiments. RU38486 was predicted to prevent the arthritis-induced rise in 11ß-OHSD activity. However on post-mortem examination it was found that the RU38486 had precipitated either around the site of subcutaneous injection or in the stomach when administered by gavage. 11ß-OHSD activity was nevertheless measured to determine whether a sufficient amount of RU38486 had remained in solution and had any effect on enzyme activity. Compared to untreated arthritic animals, 11ß-OHSD activity in liver was unexpectedly increased by administration of RU38486 in 10% DMSO (Fig. 3.7b). However, 10% DMSO alone also increased 11ß-OHSD activity, and this was not significantly different to the rise in activity observed following RU38486 administration (Fig. 3.7b). Neither 10% DMSO or RU38486 had any effect on hippocampal 11ß-OHSD activity (Fig. 3.7b). Hippocampal and hepatic 11ß-OHSD activity were unaffected by 50% ethanol and RU38486 in ethanol compared to untreated arthritic controls (Fig. 3.7a). Interestingly, RU38486 treatment led to a significant reduction in hepatic 11ß-OHSD activity when compared to ethanol treatment alone (Fig. 3.7a).

## 3.4 Discussion

Within the hippocampus, 11ß-OHSD mRNA is co-localised with both MR and GR mRNAs, supporting the view that 11ß-OHSD may regulate glucocorticoid access to corticosteroid receptors. In this chapter I have shown that glucocorticoids but not mineralocorticoids regulate 11ß-OHSD in hippocampus and liver. In addition none of the adrenal steroid manipulations had any effect on 11ß-OHSD in kidney in agreement with previous work (Smith et al., 1991).

In liver, 11ß-OHSD activity was induced by dexamethasone treatment, but in contrast was not affected by the arthritic model of stress. In contrast 11ß-OHSD activity in hippocampus was significantly increased by both glucocorticoid manipulations. A possible explanation for this discrepancy becomes apparent in the comparison of levels of activity in sham-operated controls. In liver, sham-operated control animals expressed similar levels of 11ß-OHSD activity to those seen following chronic dexamethasone treatment. Adrenalectomy led to a decrease in 11ß-OHSD in liver. In hippocampus, the opposite was found. Control animals expressed similar levels of 11ß-OHSD activity to adrenalectomised animals, while dexamethasone treatment led to a significant increase in hippocampal 11ß-OHSD activity. This therefore implies a fundamental difference in the manner in which

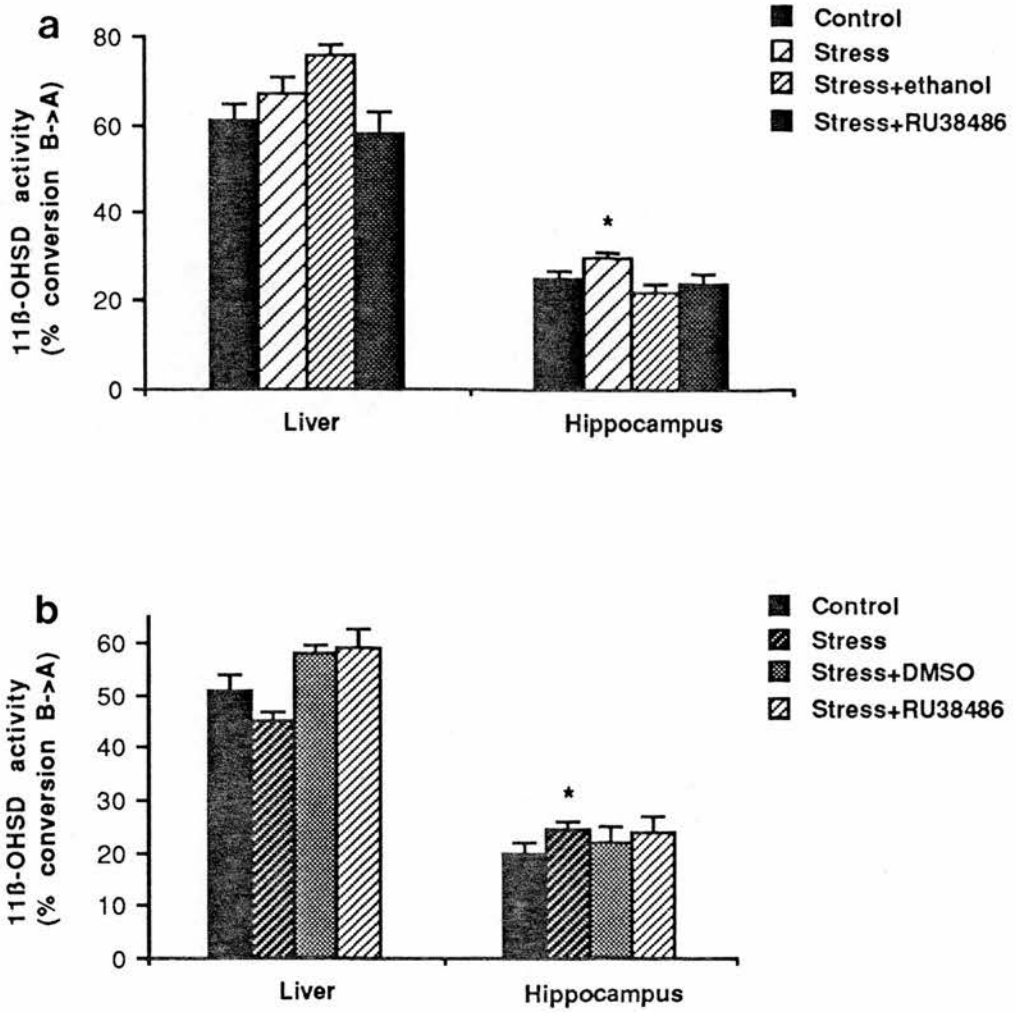



**Figure 3.7:**

Effect of 14d RU38486 treatment in (a) 50% ethanol by gavage and (b) 10% DMSO by subcutaneous injection on 11ß-OHSD activity in male rat liver and hippocampus. 11ß-OHSD activity is expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A. *$p<0.05$ compared to untreated controls. n=6/group.

hippocampus and liver are exposed to glucocorticoids under basal conditions, and that normally, 11ß-OHSD is fully induced in rat liver (possibly due to high intrahepatic glucocorticoid levels), and it is therefore not surprising that the stress model used did not alter liver enzyme activity. The difference between hepatic and hippocampal exposure to glucocorticoids may be due to the predominant direction of 11ß-OHSD activity in the two tissues. It is believed that 11ß-OHSD in liver acts as a reductase (Bush et al., 1969) such that the liver would be exposed to high levels of active glucocorticoids under basal conditions. In contrast, the direction of 11ß-OHSD activity in hippocampus is not known although a role for 11ßOHSD as a dehydrogenase has been proposed (Moisan et al., 1990a). If this were the case, 11ß-OHSD would be expected to protect the hippocampus from high glucocorticoid levels. In addition, hepatic and hippocampal 11ß-OHSD may be influenced by the presence of tissue-specific transcription factors which may regulate expression of 11ß-OHSD in a tissue-specific manner (Williams et al., 1993). However the mechanism(s) of these differences between hepatic and hippocampal 11ß-OHSD have yet to be determined.

Adrenalectomy led to an attenuation of 'liver-type 11ß-OHSD mRNA expression in hippocampus which is presumably due to a depletion of glucocorticoids since dexamethasone reversed this effect. The fact that hippocampal 11ß-OHSD activity was not attenuated, unlike mRNA expression may reflect differences in the half-lives or stability of 11ß-OHSD protein and mRNA. In support of this, sex steroid (Chapter 4) and growth hormone (Chapter 5) manipulations both show similar discrepancies between 11ß-OHSD activity and mRNA expression with mRNA changes preceding or being greater than changes in 11ß-OHSD activity. Nevertheless, northern analysis demonstrates that the glucocorticoid effects on hippocampal 11ß-OHSD activity may occur, at least in part, at the level of 11ß-OHSD gene transcription. Furthermore, sequences resembling glucocorticoid response elements have been reported in both human (Tannin et al., 1991) and rat (Moisan et al., 1992b) 11ß-OHSD genes.

Dexamethasone is a synthetic glucocorticoid which is poorly metabolised by 11ß-OHSD, and was administered in supraphysiological doses in this study. The increase in hippocampal 11ß-OHSD activity could also be caused by naturally occurring stress. Induction of arthritis was used as a model of chronically raised glucocorticoid levels, since similar rat arthritic models have been shown to chronically activate the hypothalamic-pituitary-adrenal axis (Sarlis et al., 1992). However when endogenous glucocorticoid levels were chronically, if modestly, raised following induction of arthritis (Table 3.1), 11ß-OHSD activity was significantly raised in hippocampus. This effect was prevented by prior adrenalectomy suggesting that raised glucocorticoid levels were indeed responsible for the raised hippocampal 11ß-OHSD

activity. In addition, the effects of dexamethasone and stress on hippocampal 11ß-OHSD were similar, thus indicating that the effects are likely to be directly mediated and not due to indirect actions of ACTH, since ACTH is suppressed by dexamethasone, but induced by stress. This has been further corroborated by a recent study which showed that administration of ACTH to rats had no effect on 11ß-OHSD activity in hippocampus, kidney or the vasculature (Walker et al., 1994).

Although indicative, corticosterone levels and CBG levels in the arthritic animals used in this study were not significantly different between controls and 'stress'. This may be due to one of two possibilities (i) our model of arthritis is a less severe stressor than previous arthritic models. This is supported by the lack of other adverse effects on the immune system (Donaldson et al., 1993) which may become apparent in other models (Pearson & Wood, 1959), although still leading to chronic arthritis after 14 days. (ii) It is also possible that control animals are stressed and therefore are not significantly different from the proposed 'stressed' group. Basal morning levels of corticosterone in rats are approximately 100nM or less. In this study, both control and stress groups have higher than basal corticosterone levels (337nM and 490nM respectively); it is therefore possible that control animals were stressed, although this may be related to events immediately preceding killing. Thus although the changes in hippocampal 11ß-OHSD activity with mild chronic stress were not paralleled by altered 11ß-OHSD mRNA expression, a weak trend was observed, and perhaps alternative stressors may have greater or lesser effects on 11ß-OHSD in hippocampus, but this remains to be determined.

RU38486 is a glucocorticoid antagonist which was used in these studies to determine whether the effects observed could be glucocorticoid mediated. However maintaining the antagonist in solution proved to be a problem which was not overcome. Previous studies using RU38486 often do not report how the drug was dissolved or administered (Gaillard et al., 1985), and the method of dissolving and administration of RU38486 reported in a similar study from this group (Moisan et al., 1990c) could not be reproduced here. However this study does indicate the importance of incorporating vehicle treated groups in experiments since 50% ethanol and 10% DMSO appeared to affect 11ß-OHSD activity in liver. It also indicates the difficulty faced in choosing an appropriate vehicle for compounds such as RU38486, since other similar solvents such as gossypol which is a polyphenolic compound of cotton seed oil, has recently been reported to inhibit 11ß-OHSD (Song et al., 1991).

Mineralocorticoid receptors in hippocampus are apparently non-selective, and have been shown to bind corticosterone *in vivo* (McEwen et al., 1986b). However the hippocampus may also contain aldosterone-selective mineralocorticoid receptors, suggested by the inability of corticosterone to displace all hippocampal aldosterone

binding (McEwen et al., 1986b). The selectivity mechanism in the face of excess circulating glucocorticoids has not yet been determined. In the same way as for the kidney (de Kloet & Reul, 1987; Funder, 1986), CBG was proposed as an appropriate mechanism, but since neural MR can selectively bind aldosterone in animals with little or no circulating CBG this hypothesis was rejected (Sheppard & Funder, 1987). Recently 11ß-OHSD activity has been detected in brain, with highest activities found in hippocampus and cortex (Moisan et al., 1990a; Lakshmi et al., 1991) suggesting that there may be a subpopulation of MR or GR in hippocampus which are protected, at least in part, from corticosterone by 11ß-OHSD. There is as yet, no direct experimental evidence to corroborate this mechanism in hippocampus. However co-localisation of 11ß-OHSD and MR immunoreactivities (Sakai et al., 1992) and mRNAs (this chapter) have been demonstrated providing further evidence for the involvement of 11ß-OHSD in regulating glucocorticoid access to MR and perhaps GR in hippocampus. This may prove important since either chronic excess or deficiency of glucocorticoids exert deleterious effects on the brain, including neuronal loss particularly in the hippocampus (Sloviter et al., 1989; Sapolsky et al., 1985; Sapolsky et al., 1986). Glucocorticoid-mediated regulation of 11ß-OHSD activity may function to protect sensitive neurones from long-term elevation or depression of glucocorticoid levels, such as might occur in chronic stress, affective disorders or neurodegeneration (Seckl et al., 1993). Thus corticosteroid regulation of brain 11ß-OHSD may ensure optimal exposure of neurones to glucocorticoids and preserve essential 'tonic' neurochemical functions (de Kloet & Reul, 1987; McEwen et al., 1986b), whilst not attenuating the effects of the diurnal variation, or acute stress. Although only *in vitro* 11ß-dehydrogenase activity was measured in this study, it is possible that the proposed presence of reductase activity may complicate the interpretation of these findings. Further study as to the importance of 11ß-reductase activity in brain is therefore essential to the understanding of the role of 11ß-OHSD in hippocampus, especially in light of the finding here that glucocorticoids up-regulate hippocampal 11ß-OHSD. Thus if 11ß-reductase activity predominates in hippocampus, this would lead to increased glucocorticoid exposure and the implication that 11ß-OHSD is actually a neuronal 'suicide' enzyme and not a protective mechanism as proposed.

The absence of glucocorticoid regulation in kidney may be due to high efficiency 11ß-OHSD, capable of handling even very high levels of glucocorticoids seen in stress. This would tend to indicate the need for renal 11ß-OHSD to protect MR in the distal convoluted tubules and cortical collecting ducts from prevailing glucocorticoids independent of circulating glucocorticoid concentrations (Naray-Fejes-Toth et al., 1991), thus ensuring selective access of aldosterone to renal MR even when

glucocorticoid levels are raised through chronic stress or in states of excess endogenous or exogenous glucocorticoids. Similarly, adrenalectomy would not be expected to have an effect, since the kidney would not normally be exposed to glucocorticoids due to the activity of 11ß-OHSD. A recent report has shown that 11ß-OHSD in kidney is capable of effectively metabolising dexamethasone (Siebe et al., 1993). Although metabolism of dexamethasone by hippocampal 11ß-OHSD was not looked at in this study, liver and testes, both of which express only the 'liver-type' 11ß-OHSD do not metabolise dexamethasone indicating that hippocampal 11ß-OHSD is unlikely to utilise dexamethasone as a substrate, and in addition that the distinct high activity renal 11ß-OHSD isoform may be responsible for metabolism in tissues such as kidney. Therefore the absence of regulation of 11ß-OHSD in rat kidney by dexamethasone could alternatively be explained by 11ß-OHSD metabolising dexamethasone which would then be ineffective in regulating 11ß-OHSD activity or mRNA expression. However induction of arthritis did not have any effect on renal 11ß-OHSD activity compared to controls suggesting that the inability of dexamethasone to regulate renal 11ß-OHSD cannot solely be due to its metabolism by 11ß-OHSD.

In conclusion, chronic changes in glucocorticoid, but not mineralocorticoid levels regulate 11ß-OHSD mRNA expression and enzyme activity in hippocampus and liver, but not kidney. Similarly, physiological changes in glucocorticoid levels regulate hippocampal and hepatic 11ß-OHSD activity and mRNA expression. Hippocampal 11ß-OHSD may represent a tissue-specific mechanism to ensure optimal long-term exposure of sensitive neurones to glucocorticoids.

# CHAPTER 4

# SEX STEROID REGULATION OF 11ß-OHSD

## 4.1 Introduction

11ß-OHSD activity in rats has been shown previously to exhibit a sexually dimorphic pattern of expression in both liver (Lax et al., 1978; Ghraf et al., 1975a) and kidney (Ghraf et al., 1975a; 1975b; Smith and Funder, 1991) with approximately two-fold higher activity being expressed in the male animal. This dimorphism may be due to sex steroids themselves, since studies have been performed which demonstrate that 11ß-OHSD activity is affected by the administration or withdrawal of sex steroids. For example, hepatic 11ß-OHSD activity in male rats has been shown to decrease following gonadectomy, an effect which was reversed by testosterone replacement (Lax et al., 1978). In contrast, ovariectomy appeared to have no effect on 11ß-OHSD activity in female animals, while testosterone treatment increased 11ß-OHSD activity towards the levels found in control male liver. This suggested that the normal higher level of 11ß-OHSD activity in male liver is maintained by the presence of androgens, and that the activity observed in control or ovariectomised females represents the constitutive level. This same study also showed that in contrast to, and unlike other androgen regulated enzyme activities, 11ß-OHSD activity in male and female rats was almost completely repressed by oestradiol treatment, suggesting that hepatic 11ß-OHSD activity may be oestrogen, as well as androgen regulated. Paradoxically, there was no reported change in hepatic 11ß-OHSD activity following ovariectomy, in contrast to the increase in activity which would be predicted from the removal of endogenous oestrogen.

In rat kidney the effects of sex steroids on 11ß-OHSD activity are also contradictory. In one study (Ghraf et al., 1975b), it was reported that female rats responded to ovariectomy by developing higher renal 11ß-OHSD activity similar to levels found in normal males, while castration of male animals had no effect on 11ß-OHSD activity. In addition, treatment of intact males with oestradiol resulted in a decrease in renal enzyme activity towards normal female levels, leading the authors to conclude that 11ß-OHSD activity in rat kidney was oestrogen-repressed. In a different study in contrast, the opposite was found (Smith and Funder, 1991).

Gonadectomy had no effect on 11ß-OHSD activity in male or female rat kidney, and oestradiol treatment of intact males resulted in a marked and significant increase in renal 11ß-OHSD activity.

These studies in rat liver and kidney suggest that the effects of sex steroids on 11ß-OHSD activity are tissue-specific and diverse. However the results are confusing and sometimes contradictory due to the fact that the 11ß-OHSD activity measured in these studies may result from any one of a number of isoforms of the enzyme, and the relative contributions of these isoforms to total enzyme activity was not determined. The work presented in this chapter was carried out in order to determine the effects of sex steroids on total 11ß-OHSD activity and mRNA encoding the 'liver-type' 11ß-OHSD isoform in order to investigate the relative contribution of 'liver-type' 11ß-OHSD to total 11ß-OHSD activity in liver, kidney, and also hippocampus, and to determine whether this isoform specifically is regulated by sex steroid treatments. As a natural model of high physiological levels of sex steroids, the effect of pregnancy on total 11ß-OHSD activity and 'liver-type' 11ß-OHSD mRNA expression was also investigated.

## 4.2 Results

### 4.2.1 Effect of 10d Sex Steroid Manipulations

Liver

11ß-OHSD activity exhibits sexual dimorphism in rat liver with approximately 2-fold higher enzyme activity expressed in male than female liver. Ovariectomy of female rats resulted in a marked increase in 11ß-OHSD activity (120±37% increase) which was not affected by testosterone treatment (108±24% increase compared to controls) but was reversed by oestradiol replacement (Fig. 4.1a). 10d ovariectomy resulted in a slight, but non-significant increase in 11ß-OHSD mRNA expression which was unaffected by testosterone treatment, while oestradiol replacement resulted in a significant decrease in mRNA levels (82±10% decrease compared to control levels) (Fig. 4.1b).

In male rats, gonadectomy alone or gonadectomy followed by testosterone replacement did not affect hepatic 11ß-OHSD activity (Fig. 4.2a). However, oestradiol treatment for 10d produced a large decrease in enzyme activity of 69±8% in male liver (Fig. 4.2a). In parallel, 10d oestradiol administration led to a dramatic decrease in 11ß-OHSD mRNA expression of 97±1% (Fig. 4.2b, 4.3). As with enzyme activity, no other sex steroid treatment had any effect on 11ß-OHSD mRNA expression (Figs. 4.2b, 4.3).

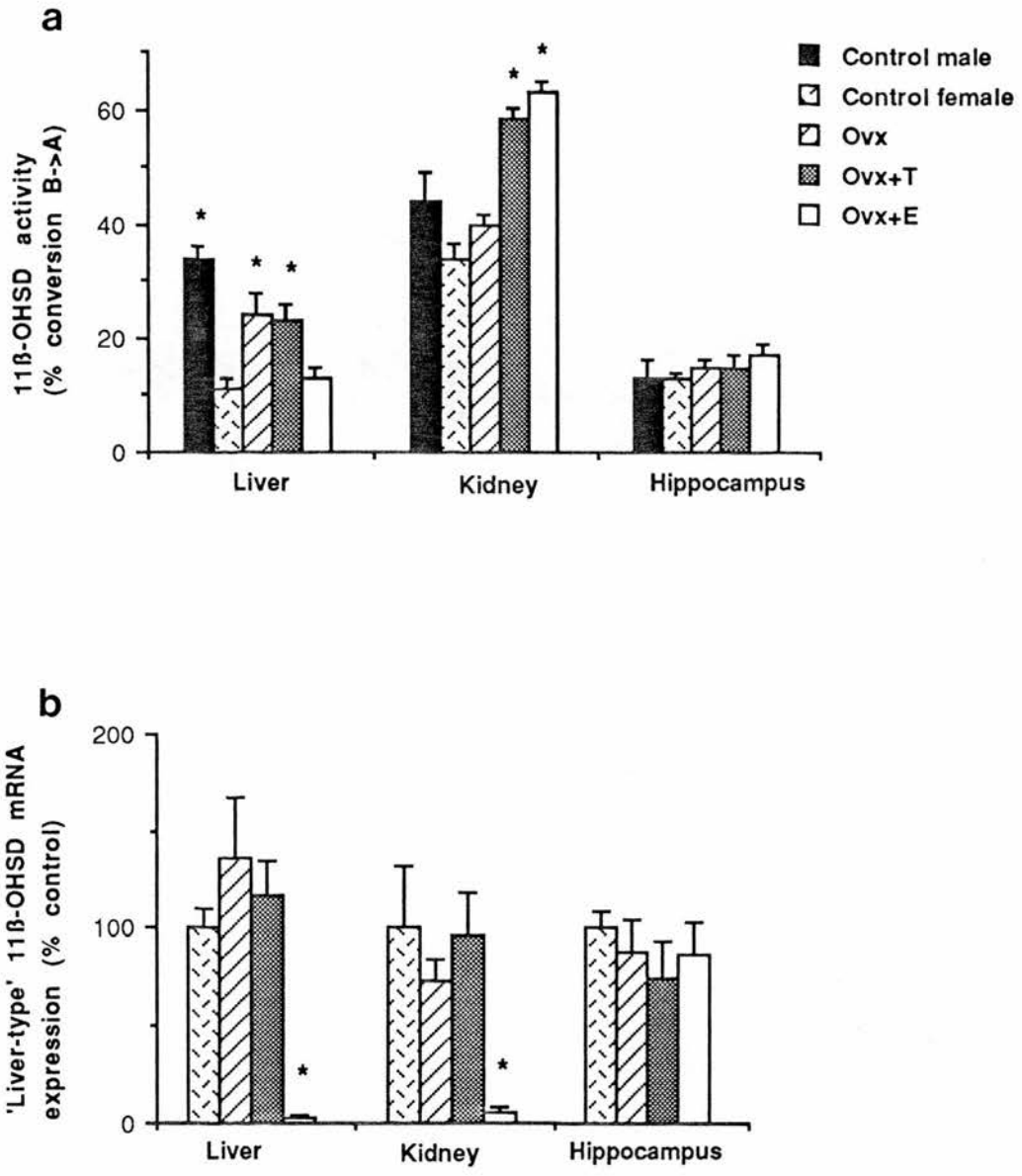



**Figure 4.1:**

Effect of 10d sex steroid manipulations on (a) 11ß-OHSD activity (expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A) and (b) 'liver-type' 11ß-OHSD mRNA expression (expressed as a percentage of mRNA levels in sham-operated control animals) in female rat liver, kidney and hippocampus. Ovx = ovariectomy; Ovx+T= ovariectomy followed by testosterone treatment; Ovx+E=ovariectomy followed by oestradiol replacement. $^*p<0.05$ compared with sham-operated control females (Duncan's Multiple Range test). n=6/group.

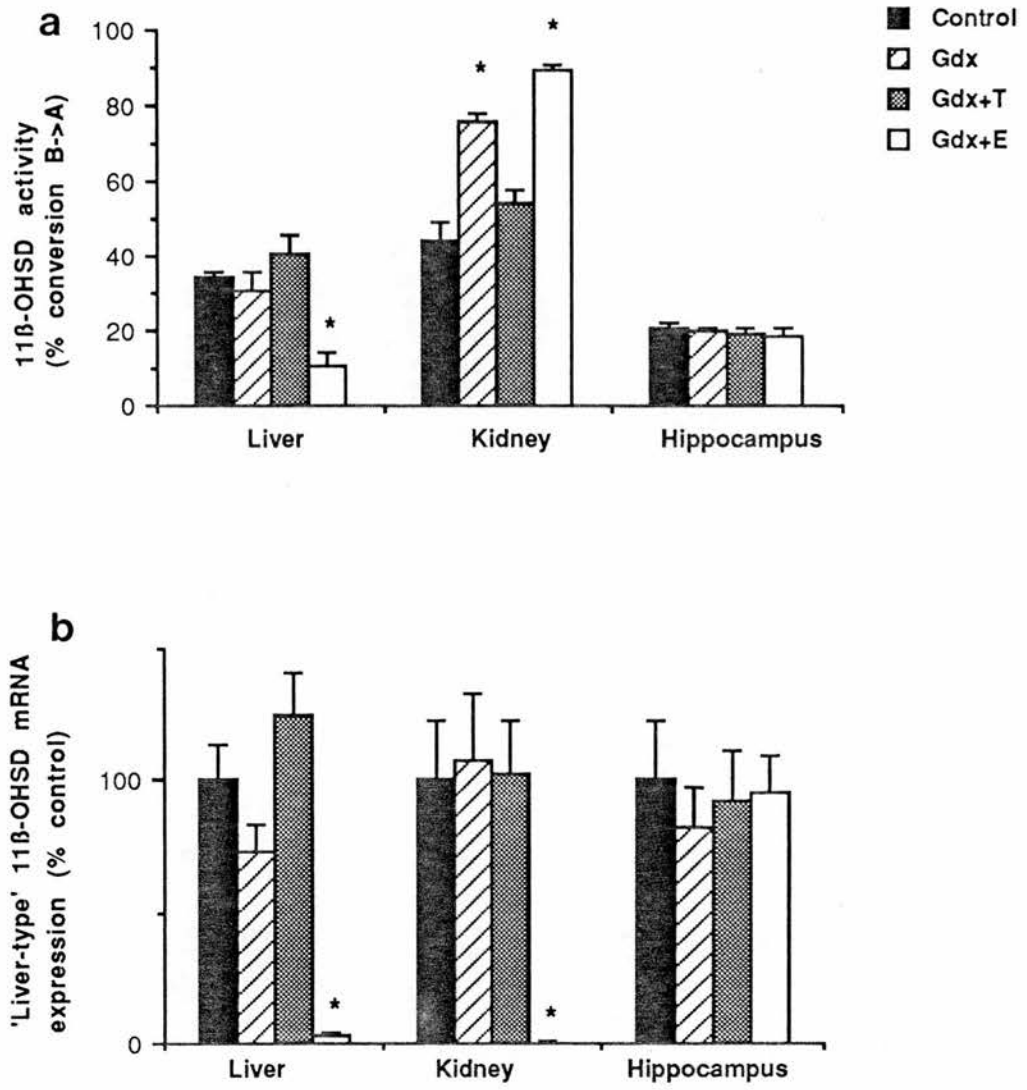



**Figure 4.2:**

Effect of 10d sex steroid manipulations on (a) 11ß-OHSD activity (expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A) and (b) 'liver-type' 11ß-OHSD mRNA expression (expressed as a percentage of sham-operated controls) in male rat liver, kidney and hippocampus. Gdx = gonadectomy; Gdx+T = gonadectomy followed by testosterone treatment; Gdx+E = gonadectomy followed by oestradiol treatment. *$p<0.05$ compared to sham-operated controls (Duncan's Multiple Range test). n=5-6/group.

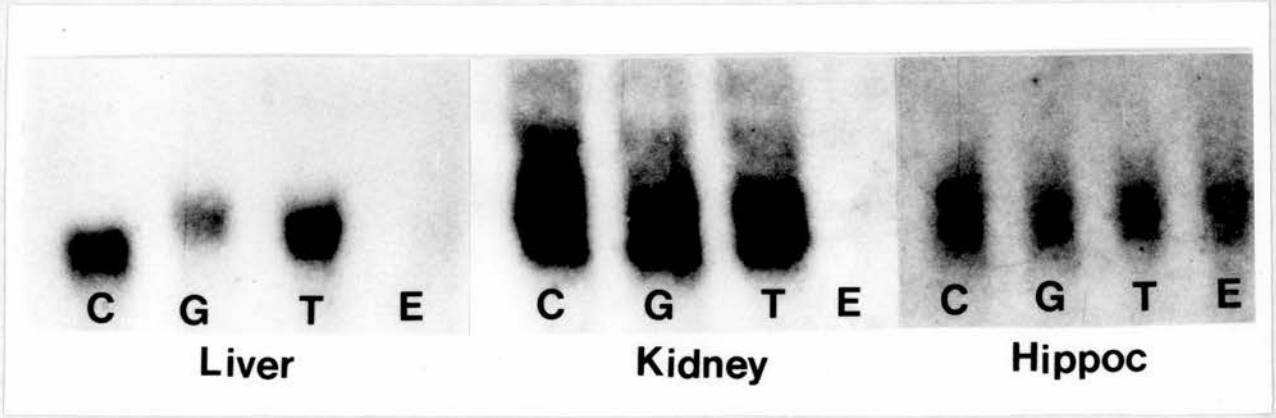



**Figure 4.3:**

Northern blot autoradiograph of 11ß-OHSD mRNA hybridised with $^{32}$P-labelled 'liver-type' 11ß-OHSD cDNA probes, showing the effects of sham-operation (C); gonadectomy (G); gonadectomy followed by testosterone replacement (T); and gonadectomy followed by oestradiol treatment (E) for 10d on 11ß-OHSD mRNA expression in male rat liver, kidney and hippocampus. Each lane represents RNA from an individual animal. 11ß-OHSD mRNA expression was quantified by computer densitometry and expressed as a ratio of the optical densities of 11ß-OHSD:7S. The 11ß-OHSD mRNA species in liver and hippocampus is 1.7 kb, while in kidney there are multiple mRNA species of 1.5, 1.6, 1.7 and 1.9 kb.

Kidney

11ß-OHSD activity in kidney does not exhibit sexual dimorphism. However, sex steroid manipulations for 10d were associated with marked changes in both 11ß-OHSD activity and mRNA expression in kidney. In female kidney, 11ß-OHSD activity was increased by 10d oestradiol replacement (91±15% rise) but not by ovariectomy either alone or following testosterone treatment (Fig. 4.1a). Similarly to liver, oestradiol treatment resulted in a dramatic decrease in 11ß-OHSD mRNA levels in female kidney (89±6% decrease) despite the induction of 11ß-OHSD activity (Fig. 4.1b).

Gonadectomy of male rats resulted in a rise in enzyme activity of 72±5% (Fig. 4.2a). Testosterone replacement prevented the gonadectomy-induced increase in 11ß-OHSD activity by 10d (Fig. 4.2a). Oestradiol treatment of gonadectomised male rats led to a large increase in 11ß-OHSD activity (103±4% rise) (Fig. 4.2a). In marked contrast, 11ß-OHSD mRNA expression was virtually undetectable after 10d (Figs. 4.2b, 4.3). It is interesting to note that all transcripts of 'liver-type' 11ß-OHSD in kidney which hybridise to the known cDNA, were suppressed by 10d oestradiol treatment (Fig. 4.3).

Hippocampus

None of the sex steroid manipulations had any effect on hippocampal 11ß-OHSD activity (Fig. 4.1a, 4.2a) or mRNA expression (Figs. 4.1b, 4.2b, 4.3) in male or female rats at 10d.

### 4.2.2 In Situ Hybridisation

Ten days after gonadectomy 11ß-OHSD mRNA was highly expressed in the male kidney, showing the same localisation predominantly in the inner cortex and outer medulla as has been previously shown in control male kidney (Yau et al., 1991). 10d oestradiol treatment of gonadectomised male rats markedly attenuated 11ß-OHSD mRNA expression in all kidney regions (Fig. 4.4).

### 4.2.3 Effect of 48h Sex Steroid Manipulations

The oestrus cycle in the rat extends over approximately four to five days. Therefore as an exaggerated model of the oestrus cycle, and as a time course of the effects of sex steroids on 11ß-OHSD activity and 'liver-type' mRNA expression, both were measured in male rats following sex steroid manipulations for 48h.

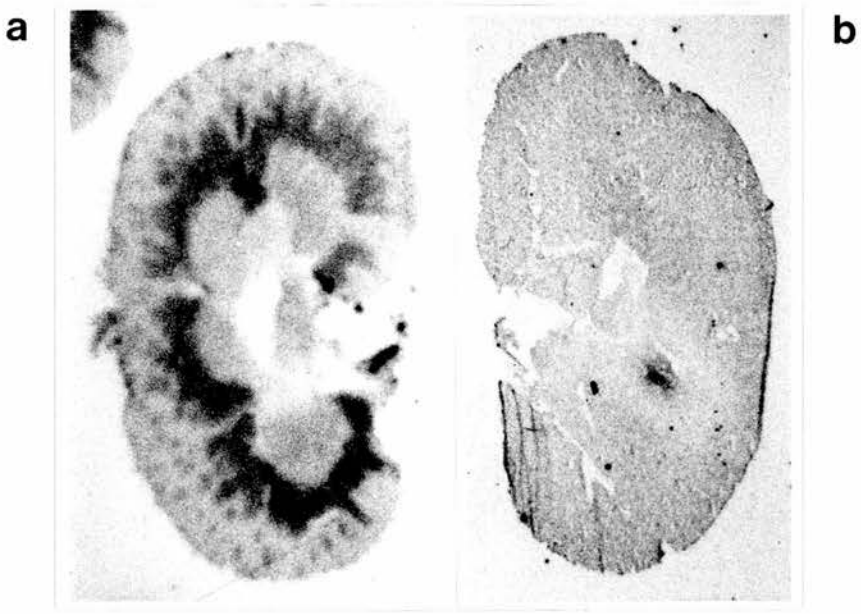



**Figure 4.4:**

Autoradiograph of longitudinal sections of male Han Wistar rat kidney hybridised with $^{35}$S-labelled cRNA probes showing 'liver-type' 11ß-OHSD mRNA expression following (a) gonadectomy and (b) gonadectomy + oestradiol treatment for 10d.

Liver

48h gonadectomy alone, and gonadectomy with testosterone replacement or oestradiol treatment, had no effect on 11ß-OHSD activity in male liver (Fig. 4.5a). Similarly, 48h gonadectomy alone or with testosterone replacement had no effect on hepatic 11ß-OHSD mRNA expression in males. However, 48h gonadectomy with oestradiol treatment led to a significant reduction of 11ß-OHSD mRNA expression by 77±7% (Fig. 4.5b) despite having no effect on enzyme activity at this time point.

Kidney

Sex steroid manipulations were associated with marked changes in both 11ß-OHSD enzyme activity and mRNA expression at 48h in male kidney. Gonadectomy resulted in a small, non-significant rise (21±7%) in enzyme activity at 48h (Fig. 4.5a), which was unaffected by testosterone replacement (46±7% rise compared to controls; Fig 4.5a). Gonadectomy led to a parallel increase in kidney 11ß-OHSD mRNA levels at 48h (72±5% rise; Fig. 4.5b), which again was not reversed by 48h testosterone replacement (Fig. 4.5b).

Oestradiol treatment of gonadectomised male rats resulted in a large increase in 11ß-OHSD activity (82±1% rise) at 48h (Fig. 4.5a). In striking contrast, 11ß-OHSD mRNA expression was markedly attenuated by 48h oestradiol treatment (decrease of 84±4%; Fig. 4.5b). In the same way as 10d oestradiol treatment in kidney, all transcripts of the 'liver-type' 11ß-OHSD which hybridise to the known cDNA were suppressed by 48h oestradiol treatment (data not shown).

Hippocampus

None of the sex steroid manipulations had any effect on hippocampal 11ß-OHSD activity (Figs. 4.5a) or mRNA expression (Fig. 4.5b) at 48h in male rats.

### 4.2.4 11ß-OHSD Activity During the Oestrus Cycle

Normal female adult Han Wistar rats were smeared daily, and cervical cells examined microscopically to determine whether each animal was in pro-oestrus, oestrus or met-oestrus. Rats were then divided into three groups based on the three stages of the oestrus cycle. 11ß-OHSD activity was measured in liver, kidney and hippocampus of these groups of animals, and was found to be unaffected by the oestrus cycle (Fig. 4.6).

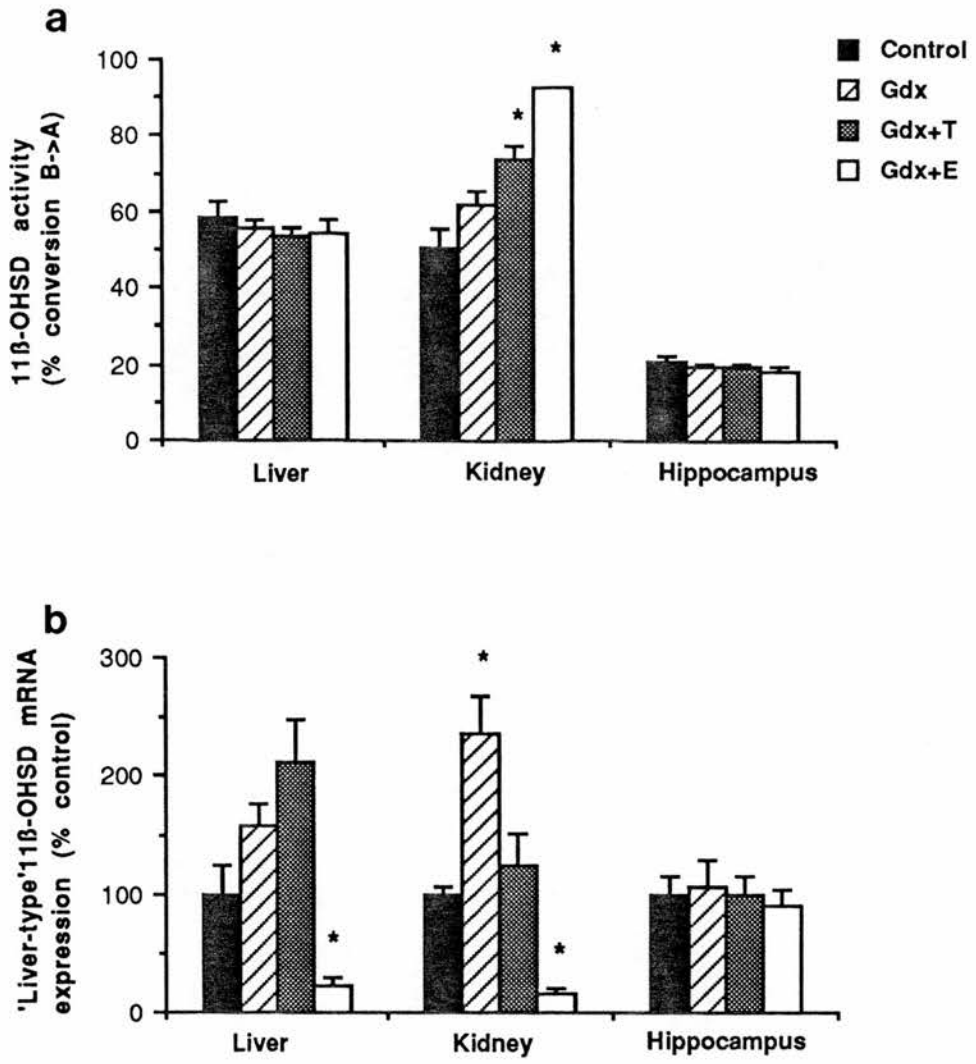



**Figure 4.5:**

Effect of 48h sex steroid manipulations on (a) 11ß-OHSD activity (expressed as percentage conversion [$^3$H]-B to [$^3$H]-A, and (b) 'liver-type' 11ß-OHSD mRNA expression (expressed as a percentage of levels in sham-operated controls) in male rat liver, kidney and hippocampus. Gdx = gonadectomy; Gdx+T = gonadectomy followed by testosterone replacement; Gdx+E = gonadectomy followed by oestradiol treatment. *$p<0.05$ compared with sham-operated controls (Duncan's Multiple Range test). n=5-6/group.

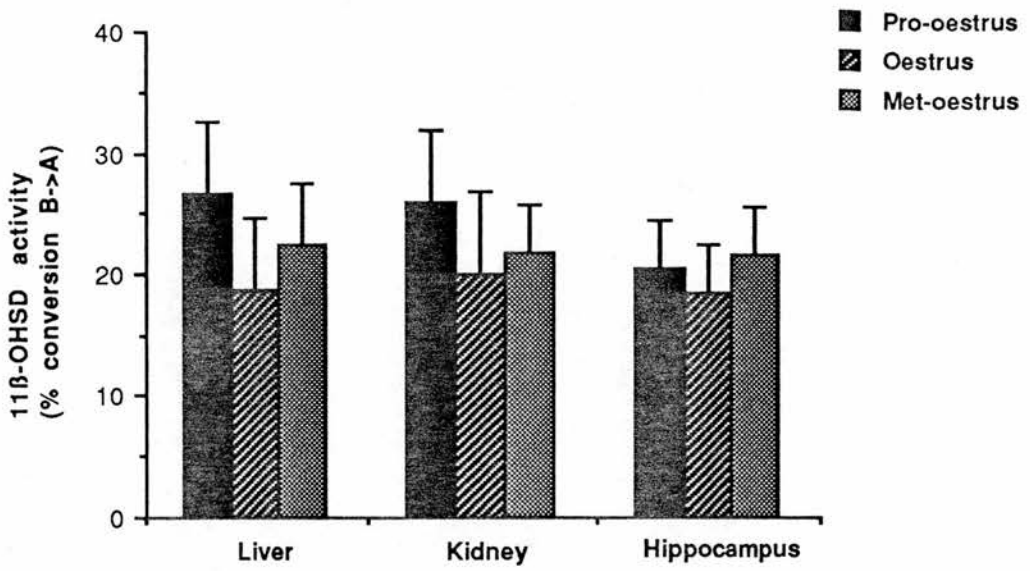



**Figure 4.6:**

11ß-OHSD activity during the oestrus, met oestrus and di-oestrus stages of the oestrus cycle in female rat liver, kidney and hippocampus. Data are expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A. *$p<0.05$ (Duncan's Multiple Range test). n=5/group.

### 4.2.5 11ß-OHSD in Pregnancy

On day 19 of pregnancy, when oestrogen levels in the rat are raised (Albert et al., 1992), 11ß-OHSD activity in liver is significantly higher (46±2% higher) than the activity measured in non-pregnant control female rats (Fig. 4.7a) in contrast to the repression of 11ß-OHSD activity observed after 10d oestradiol treatment of male and female animals (Figs. 4.3a, 4.5a). Similarly, 11ß-OHSD mRNA expression was also significantly raised in pregnancy (277±87% increase) compared to non-pregnant controls (Fig. 4.7b).

Renal 11ß-OHSD activity was significantly decreased by day 19 of pregnancy (43±7% decrease), again in contrast to the marked induction in 11ß-OHSD activity observed following gonadectomy with oestradiol treatment of male or female rats (Fig. 4.7a). However, despite the repression of renal 11ß-OHSD activity during pregnancy, 'liver-type' 11ß-OHSD mRNA levels in kidney were unaffected by pregnancy (Fig. 4.7b).

## 4.3 Discussion

In agreement with previous studies, I have demonstrated that 11ß-OHSD exhibits a sexually dimorphic pattern of expression in rat liver (Lax et al., 1978; Ghraf et al., 1975a), and that 11ß-OHSD activity is regulated in liver and kidney in a tissue-specific manner by chronic sex steroid treatments (Lax et al., 1978; Smith et al., 1991). I have also demonstrated that the mRNA encoding the 'liver-type' 11ß-OHSD mRNA is regulated by the sex steroid manipulations employed and have provided further evidence for the existence of a second 11ß-OHSD isoform which is differentially regulated by oestradiol treatment in rat kidney. Finally, using pregnancy as a natural model of high oestrogen levels, I have shown that total 11ß-OHSD activity and 'liver-type' 11ß-OHSD mRNA expression are regulated during pregnancy in a tissue-specific manner, but interestingly, not in the manner predicted by the results of the chronic oestradiol manipulation studies.

11ß-OHSD activity in liver in female rats is approximately 50% of the levels of activity exhibited in male rat liver (Fig. 4.1a). However 10d ovariectomy resulted in a two-fold increase in 11ß-OHSD activity which was reversed by oestradiol replacement but not testosterone treatment Similarly in male rat liver, 10d gonadectomy either alone or with testosterone replacement had no effect on 11ß-OHSD activity while oestradiol treatment for 10d following gonadectomy repressed male hepatic 11ß-OHSD activity to levels similar to those expressed in control female liver. These results therefore suggest that the lower levels of 11ß-OHSD activity

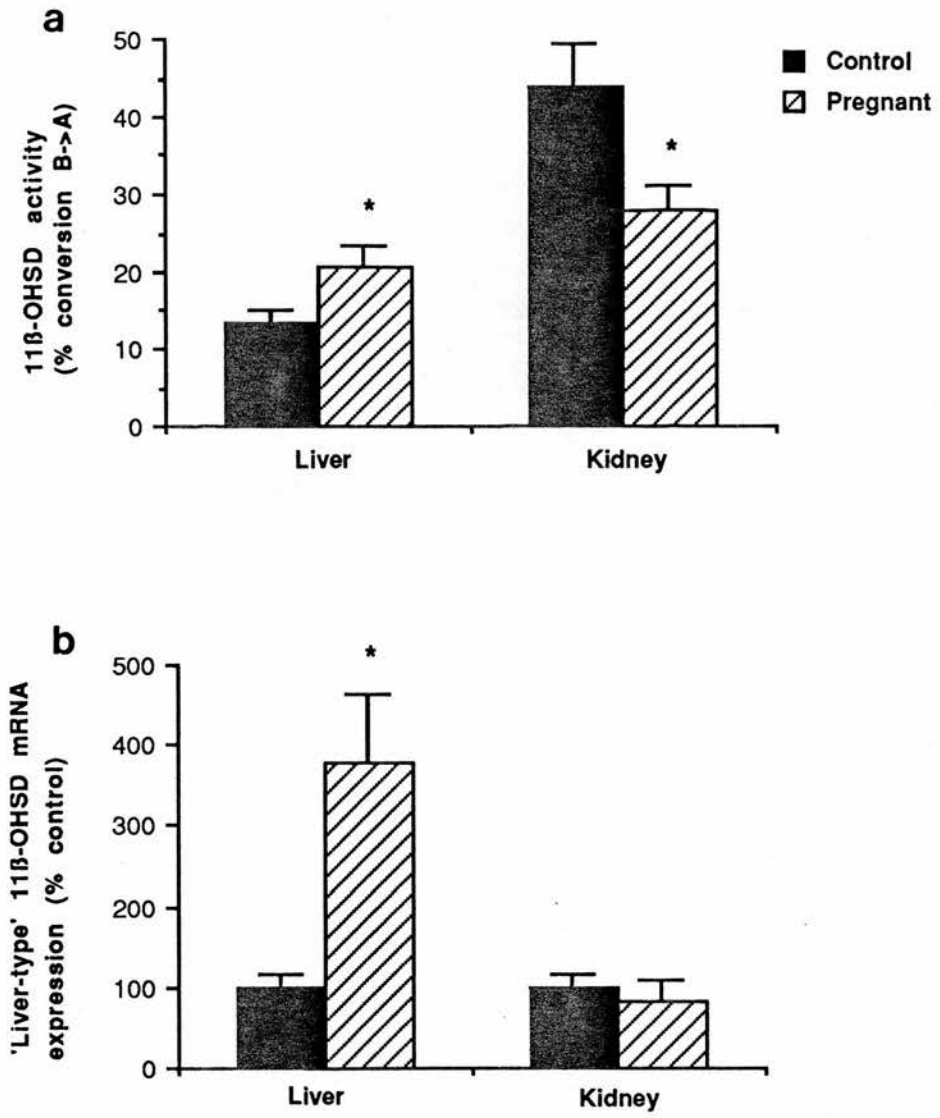



**Figure 4.7:**

(a) 11ß-OHSD activity (expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A) and (b) 'liver-type' 11ß-OHSD mRNA expression (expressed as a percentage of non-pregnant controls) during pregnancy in female rat liver and kidney. $^*p<0.05$ compared with non-pregnant controls (Duncan's Multiple Range test). n=7/group.

expressed in normal female liver are due to oestrogen repression of the enzyme. 'Liver-type' 11ß-OHSD mRNA levels in liver show the same trend as total 11ß-OHSD activity although the changes in female animals do not reach significance. Remarkably, although 11ß-OHSD activity is only repressed to control female levels by the oestradiol manipulations, 'liver-type' 11ß-OHSD mRNA expression is drastically decreased by this treatment in both male and female rats. Perhaps the 11ß-OHSD activity would also decrease further in parallel to mRNA levels, but the treatment period was not extended to determine if this was indeed the case. However at 48h, gonadectomised male animals treated with oestradiol, exhibited significantly decreased levels of 'liver-type' 11ß-OHSD mRNA whereas total activity was not effected suggesting that 11ß-OHSD mRNA and protein may have different half-lives. Alternatively, there is the possibility of a different isoform of 11ß-OHSD being responsible for the activity measured, or it may be simply that the enzyme assay is not as sensitive in detecting perturbations in 11ß-OHSD activity as northern analysis is at revealing changes in mRNA levels, or that mRNA levels although undetectable, are not truly zero. Irrespective of the reasons for the different degrees of effect on 11ß-OHSD activity and mRNA expression, it is apparent from the marked repression of 'liver-type' 11ß-OHSD mRNA levels that although published data suggested that the dose of oestradiol administered in these experiments would result in plasma oestrogen levels not significantly different from those of normal female rats (Painson et al., 1992), this is not the case here. It is likely that the oestrogen levels produced in these animals are higher than levels found in control females, which led to repression of 'liver-type' 11ß-OHSD mRNA below levels expressed in control females. In these experiments the levels of plasma oestrogen were not measured, but obviously this would provide useful information in any future studies.

In both male and female rat kidney, oestradiol treatment at 48h and 10d had strikingly different effects on total 11ß-OHSD activity and 'liver-type' 11ß-OHSD mRNA expression. While oestradiol treatment markedly induced 11ß-OHSD activity, the same treatment almost completely repressed 'liver-type' 11ß-OHSD mRNA expression in both male and female kidney. There are several possible explanations for the discrepancies between 11ß-OHSD activity and mRNA expression in kidney. (i) This might represent differences in turnover/half-life of mRNA and protein. However given the extent of the suppression of 'liver-type' 11ß-OHSD mRNA, it is very unlikely that increased enzyme activity represents increased translation of any remaining mRNA. In addition, the hepatic 11ß-OHSD data in which enzyme activity decreased broadly in parallel to 'liver-type' mRNA expression by 10d suggest that gross differences between mRNA and protein half-life seem unlikely explanations for the opposite changes in mRNA levels and enzyme activity

seen in the kidney. (ii) Previous studies using 11ß-OHSD cDNA probes encoding the 'liver-type' 11ß-OHSD have shown multiple RNA species in the kidney (Krozowski et al., 1990), one of which encodes a truncated protein which contains the putative active sites of the enzyme. However it appears unlikely that any one of these mRNA species is responsible for the high enzyme activity in the kidney since all result from differential promoter usage of the same gene (Moisan et al., 1992b) and all were repressed following oestradiol treatment. (iii) Alternatively, a second gene product might be responsible for the oestradiol-induced renal 11ß-OHSD enzyme activity. The second gene product would presumably produce mRNA which hybridises very weakly or not at all on northern analysis with the cloned 'liver-type' 11ß-OHSD cDNA. This would be consistent with a growing body of evidence suggesting the presence of at least one other distinct 11ß-OHSD isoform (described in section 1.3.5).

Gonadectomy of male rats caused a transient increase in renal 11ß-OHSD activity and mRNA expression at 48h, although the increase in activity did not reach significance. Testosterone replacement for 48h reversed the increase in 11ß-OHSD mRNA levels, but did not affect enzyme activity. However at 10d, the increase in 11ß-OHSD activity as a result of gonadectomy persisted, and testosterone replacement reversed the effect of gonadectomy on 11ß-OHSD activity. These results again indicate a possible difference in half-life of 11ß-OHSD protein and mRNA, although sex steroid effects on renal 11ß-OHSD may also be complicated by the presence of a second isoform of 11ß-OHSD.

The oestrus cycle in the rat extends over four to five days and three stages - pro-oestrus, oestrus, and met-oestrus. These studies have demonstrated that 11ß-OHSD activity in female liver, kidney and hippocampus does not alter during the oestrus cycle suggesting that physiological cyclical variations in oestrogen levels in females do not perturb 11ß-OHSD enzyme activity. This parallels the effects of 48h sex steroid manipulations, although the effects of the oestrus cycle on 'liver-type' mRNA expression have not been demonstrated.

In contrast to liver (and kidney), hippocampal 11ß-OHSD activity and mRNA expression were unaffected by any of the sex steroid manipulations employed. The difference is unlikely to be due to oestradiol or testosterone not gaining access to the brain, since both steroids readily cross the blood-brain barrier and bind to central receptors when administered peripherally. Furthermore, although documented tissue-specific promoter usage of the 11ß-OHSD gene (Moisan et al., 1992b) might explain the absence of sex steroid regulation in the hippocampus, work in our laboratory has shown that liver and hippocampus predominantly employ the same promoter (Low et al., 1993), making this an unlikely explanation for the differences in the regulation of 11ß-OHSD in the two tissues. However sexually dimorphic expression of many

genes and gene products is thought to be due to an indirect effect of sex steroids acting on the secretory profiles of growth hormone, which differ in male and female rats (Saunders et al., 1976; Tannenbaum & Martin 1976) as described in section 1.3.8. Furthermore, Lax et al.(1978) have shown that the actions of oestradiol on 11ß-OHSD activity require an intact pituitary. Possible regulation of 11ß-OHSD by growth hormone (GH) has been investigated and described in Chapter 6. The lack of regulation of hippocampal 11ß-OHSD by sex steroids would therefore reflect a failure of peripheral growth hormone, as with many other peptides, to cross the blood-brain barrier, and gain access to the brain.

There are obviously several discrepancies between this, and previous studies of the role of sex steroids in the sexual dimorphism of 11ß-OHSD in rat liver and kidney. Part of the reason for this may be that Lax et al. (1978) and Ghraf et al. (1975a; 1975b) were looking at regulation of 11ß-OHSD activity in liver and kidney microsomes using cortisol as substrate and NAD and co-substrate at pH 9.5. However, cortisol is not an appropriate substrate for rat 11ß-OHSD since the physiological glucocorticoid in the rat is corticosterone. In addition NAD is the preferred co-factor for 11ß-OHSD 2 of which there is no evidence in liver, and although 11ß-OHSD 2 is found in kidney, it is not exclusively found in microsomes (Brown et al., 1993a). Finally, pH 9.5 is approximately optimal for looking at 'liver-type' 11ß-OHSD, while 11ß-OHSD 2 prefers more physiological pH (Brown et al., 1993a). Thus due to quite different experimental procedures, meaningful comparisons of results between this and previous studies cannot reliably be made.

I also examined the effect of high physiological oestradiol levels during pregnancy on 11ß-OHSD activity and mRNA expression. Pregnancy is a natural model for chronically raised oestradiol levels (Albert et al., 1992), together with increased levels of many other steroid hormones. During pregnancy, 11ß-OHSD activity and mRNA expression in liver are both significantly raised in contrast to the effects of chronic oestradiol treatment in either male or female animals. Similarly, renal 11ß-OHSD activity following gonadectomy and oestradiol treatment was markedly increased, whereas during pregnancy it was significantly reduced. Although this experiment was intended as a physiological model of raised oestrogen levels to corroborate results of pharmacological oestrogen manipulations, opposite directions of regulation were found in both experiments. Thus other factors in pregnancy in addition to oestrogen must regulate 11ß-OHSD. Progesterone levels are also much higher during pregnancy (Albert et al., 1992), and progesterone is known to inhibit 11ß-OHSD, at least in placenta (Lopez-Bernal et al., 1980; Murphy, 1981). Therefore manipulation of progesterone levels is probably the most obvious way to begin looking at regulation of 11ß-OHSD during pregnancy. It is also interesting that

11ß-OHSD activity in kidney is reduced during pregnancy. Inhibition of 11ß-OHSD by GE has been shown to result in *in vivo* binding of [$^3$H] corticosterone in the kidney in a manner indistinguishable from [$^3$H] aldosterone binding (Edwards et al., 1988). It is possible then, that decreased 11ß-OHSD activity during pregnancy could lead to increased exposure of renal mineralocorticoid receptors to glucocorticoids possibly adding to the incidence of plasma volume expansion and even high blood pressure often seen during pregnancy. There are several ways in which the role of 11ß-OHSD in plasma volume expansion and raised blood pressure during pregnancy could be considered. However, blood pressure measurements in rats are technically difficult to do, and measurement of blood volume changes in pregnancy would be inappropriate since blood volume increases in normal pregnancy anyway. Nevertheless, it should be possible to look at the role of 11ß-OHSD in protecting renal mineralocorticoid receptors from exposure to glucocorticoids during pregnancy by measuring [$^3$H] corticosterone binding in kidney by *in vivo* autoradiography following administration of tracer amounts of [$^3$H] corticosterone to previously adrenalectomised pregnant and non-pregnant control rats.

In summary, the studies in this chapter demonstrate that 11ß-OHSD is regulated in a tissue-specific manner by sex steroids. In addition, hepatic 11ß-OHSD exhibits sexual dimorphism with higher 11ß-OHSD expressed in male than female liver. The discrepancy between oestradiol-mediated induction of 11ß-OHSD activity in the kidney in the face of almost total repression of the cloned 'liver-type' gene provide strong support to the growing body of evidence indicating the existence of more than one gene encoding 11ß-OHSD activities. The nature of the oestrogen-induced renal enzyme activity remains to be determined. The possibility that oestradiol regulation of 11ß-OHSD is due, at least in part, to growth hormone, is explored in the following chapter.

# CHAPTER 5

# GROWTH HORMONE REGULATION OF 11ß-OHSD

## 5.1 Introduction

In Chapter 4, I demonstrated that hepatic 11ß-OHSD is sexually dimorphic with the higher enzyme activity found in male animals. Ovariectomy of normal females led to an increase in 11ß-OHSD activity and mRNA expression towards control male levels. This effect was reversed by oestradiol replacement but not affected by testosterone treatment. In this chapter I examine the mechanism by which sex steroids exert this sexually dimorphic expression on 11ß-OHSD.

Many hepatic genes and gene products which typically exhibit a sexually dimorphic pattern of expression are thought to be regulated indirectly by sex-specific profiles of growth hormone (GH) from the pituitary (Gustafsson et al., 1983). Plasma levels of GH in male and female rats are very similar although the secretory profiles show significant differences between the sexes. In adult male rats, basal GH secretion is very low but is interspersed by infrequent, high amplitude spikes. In contrast, in the female animal GH patterns are more continuous with higher basal levels of GH, and secretory pulses which are more frequent but of lower amplitude (Saunders et al., 1976; Tannenbaum & Martin, 1976). In this chapter I address the possibility that sex steroid effects on 11ß-OHSD are indirect, and mediated sex steroid-specific patterns of GH release.

Lax et al. (1978) have previously demonstrated that hypophysectomy abolishes the sexual dimorphism of hepatic 11ß-OHSD by increasing enzyme activity in females to typical male levels indicating that the hypophysis has a repressive effect on hepatic 11ß-OHSD activity in females. Testosterone or oestradiol treatment of hypophysectomised animals had no effect on hepatic 11ß-OHSD leading the authors to suggest that the hypophysis may have a permissive effect on the influence of sex steroids on hepatic 11ß-OHSD. Any possible active involvement of the pituitary in maintaining sexual dimorphism of 11ß-OHSD in rat liver was not investigated. Similarly, Ghraf et al (1975b), showed that 11ß-OHSD activity in rat kidney was oestrogen repressed in female animals, and the low levels of renal 11ß-OHSD could be increased towards control male levels by ovariectomy or hypophysectomy. These

authors did not however, examine the effects of sex steroids or pituitary hormone replacement on renal 11ß-OHSD following hypophysectomy.

In this chapter I describe experiments on the regulation of 11ß-OHSD by GH in a rat model of selective GH deficiency. This particular strain of GH deficient (dwarf) rats are homozygous for an autosomal recessive mutation which reduces pituitary GH to 5% of normal, and plasma GH to barely detectable levels (<5ng/ml) (Charlton et al., 1988). The mutation originally arose spontaneously and is thought to result in insufficient GH production by the pituitary while there is no evidence for reduced GRF or enhanced SS production. Similarly, leutenising hormone, prolactin, thyroid stimulating hormone, oxytocin, vasopressin and gonadal steroids are not significantly affected in the dwarf animals compared to their heterozygous litter mates. In addition, GH treatment of the homozygous dwarf results in significantly increased growth rates and longitudinal bone growth (Charlton et al., 1988). However these dwarf rats are an inbred genetic strain which may exhibit additional unexpected abnormalities that may affect these studies. To overcome this potential problem, and to further study the possible effects of reduced GH levels in these rats, GH and/or oestradiol treatments following hypophysectomy have also been examined.

## 5.2 Results

### 5.2.1 Growth Hormone Regulation of 11ß-OHSD in Dwarf Rats

Liver

In these experiments, the control rats used were the NIMR/AS strain which is the parent strain from which the dwarf rats were bred. 11ß-OHSD activity in normal (AS) rat liver exhibits sexual dimorphism, with the higher enzyme activity being expressed in male liver (50% higher than normal females) (Fig. 5.1a). There was a smaller sex difference in dwarf rats, although dwarf males still expressed significantly higher hepatic 11ß-OHSD activity (36% higher than dwarf females). However both sexes of dwarf rats had similar levels of hepatic 11ß-OHSD activity to normal males (Fig. 5.1a). Similarly, hepatic 11ß-OHSD mRNA levels were lower in normal females (72% lower compared to normal males), while 11ß-OHSD mRNA levels in dwarf male and female liver were not significantly different, although there may be a trend towards lower expression in female dwarf rats (Fig. 5.1b, 5.2).

Treatment of dwarf male animals with continuous (female pattern) GH (200µg/day by mini osmotic pumps) for 6d led to a significant decrease in 11ß-OHSD activity (30% decrease) towards, to levels similar to those found in dwarf females (Fig. 5.3a). In contrast, 6d pulsatile (male pattern) GH treatment (25µg every

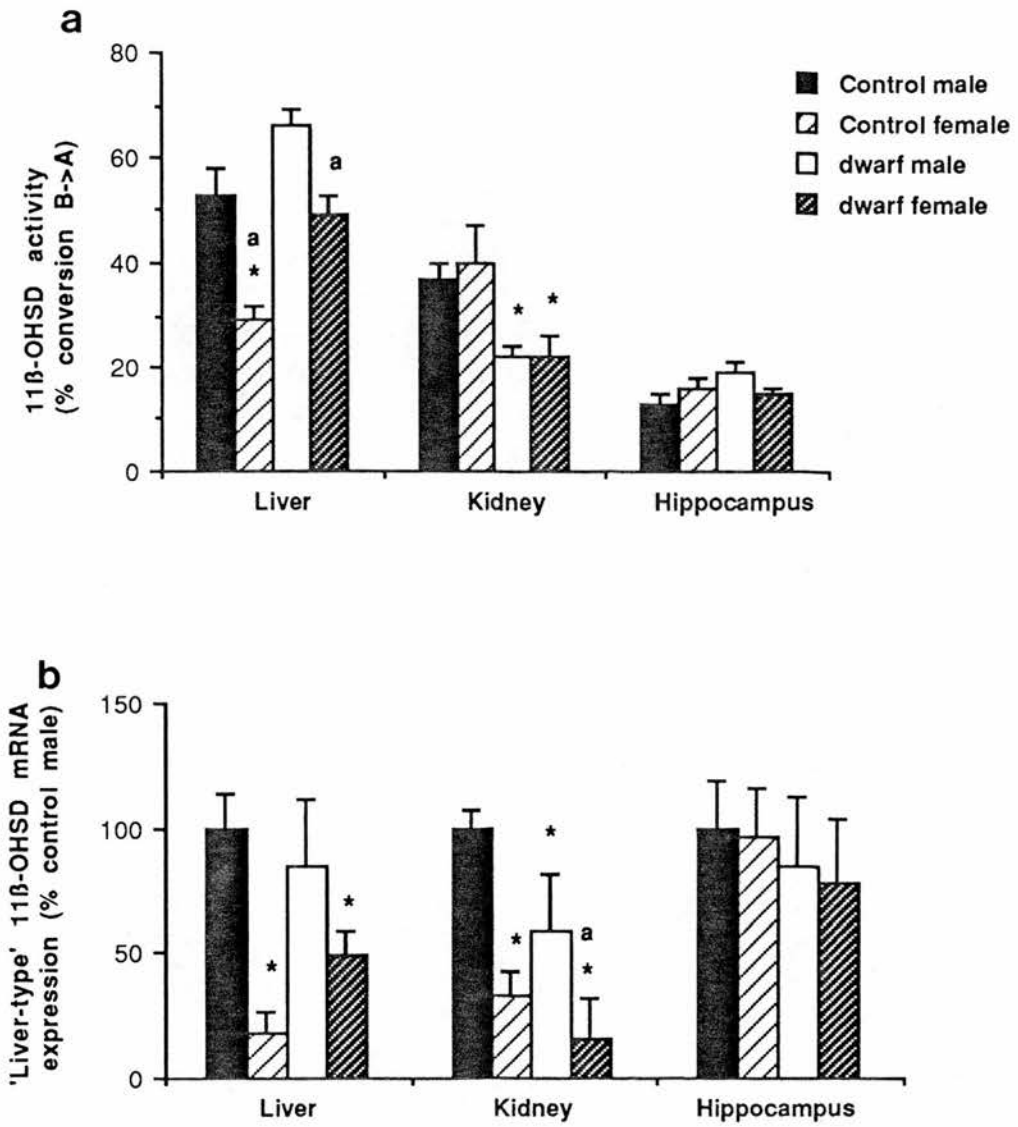



**Figure 5.1:**

(a) 11ß-OHSD activity (expressed as percentage conversion [$^3$H]-B to [$^3$H]-A) and (b) 'liver-type' 11ß-OHSD mRNA expression (expressed as a percentage of normal male levels) in normal and dwarf male and female rat liver, kidney and hippocampus. $^*p<0.05$ compared with normal males, $^ap<0.05$ compared with dwarf males (Duncan's Multiple Range test). n=6/group.

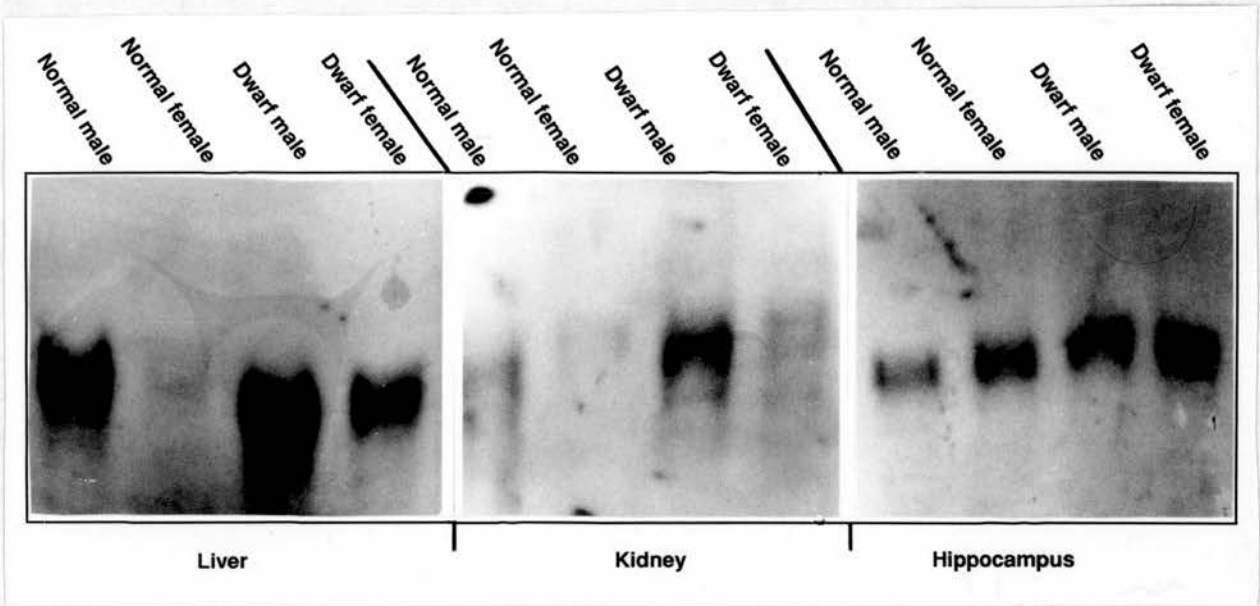



**Figure 5.2:**

Northern blot autoradiograph of 'liver-type' 11ß-OHSD mRNA expression in normal and dwarf male and female rat liver, kidney and hippocampus. Blots were hybridised with $^{32}$P-labelled 11ß-OHSD cDNA probes. Each lane represents total RNA from an individual animal. 11ß-OHSD mRNA expression was quantified by computer densitometry and expressed as a ratio of the optical densities of 11ß-OHSD:7S. The 11ß-OHSD mRNA species in liver and hippocampus is 1.7 kb, while in kidney there are multiple mRNA species of 1.5, 1.6, 1.7 and 1.9 kb.

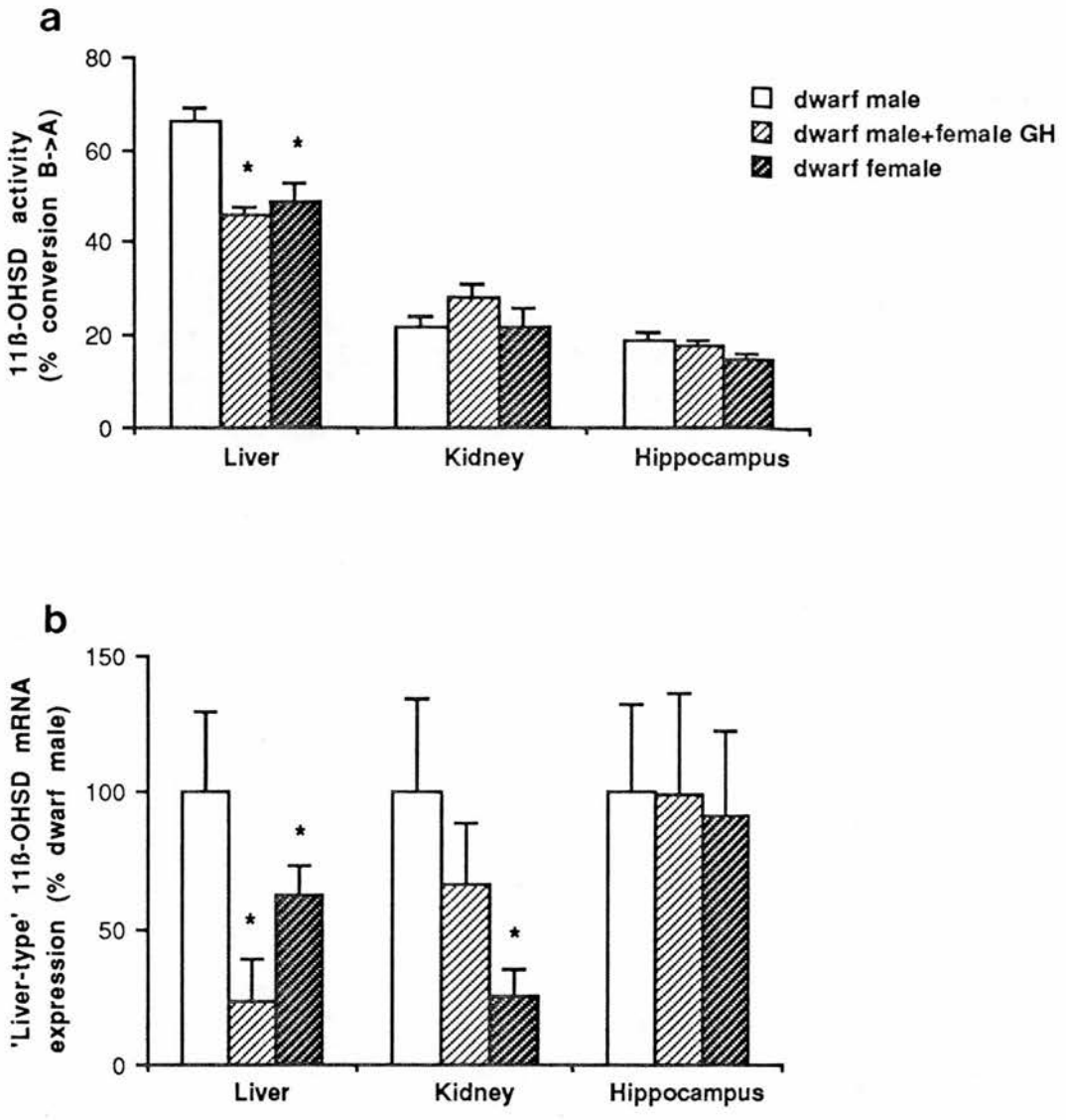



**Figure 5.3:**

Effect of 6d continuous (female pattern) GH on (a) 11ß-OHSD activity (expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A) and (b) 'liver-type' 11ß-OHSD mRNA expression (expressed as a percentage of dwarf male levels) in male dwarf rats. *$p<0.05$ compared to dwarf male levels (Duncan's Multiple Range test). n=5/group.

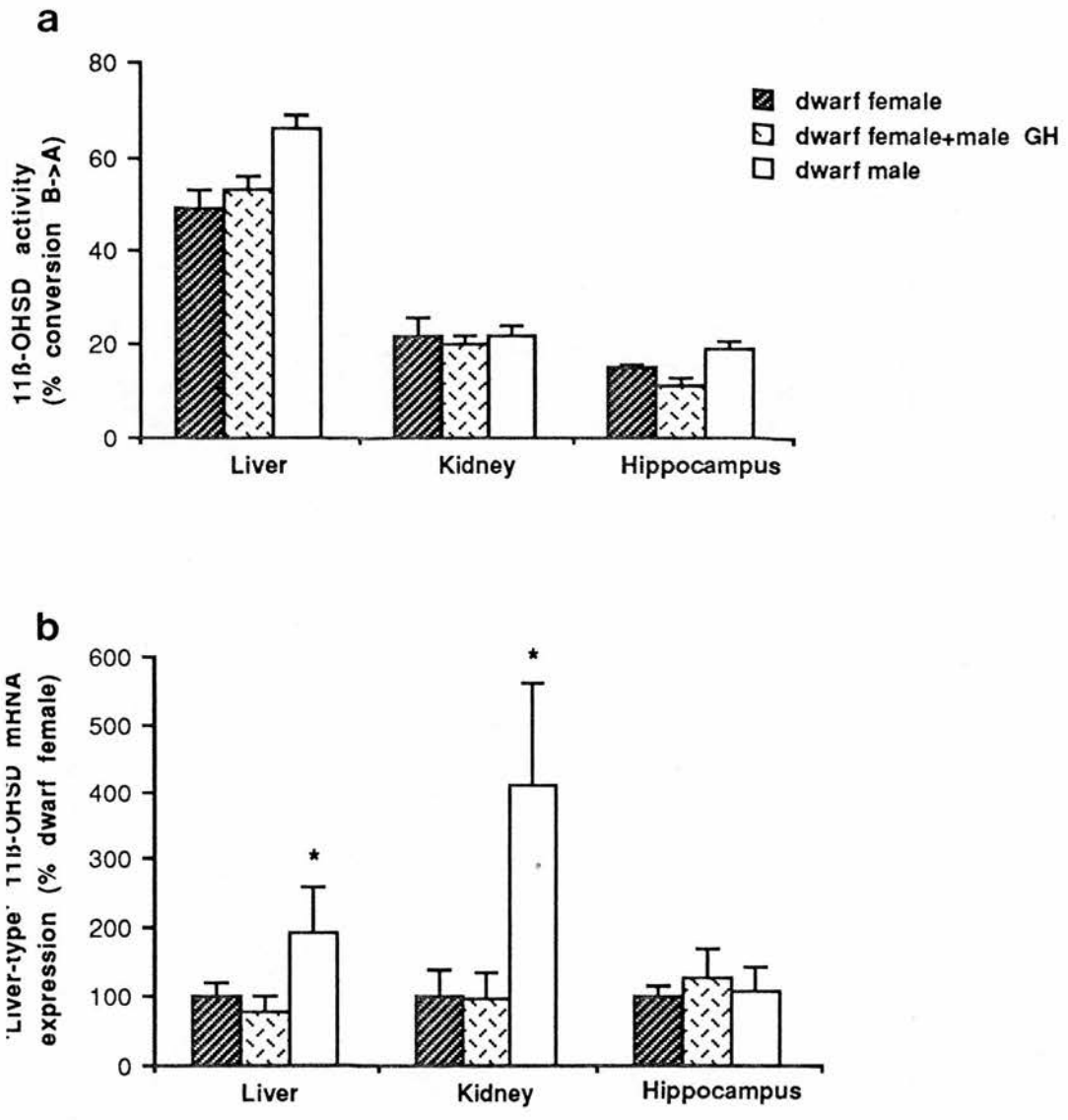



**Figure 5.4:**

Effect of 6d pulsatile (male pattern) GH on (a) 11ß-OHSD activity (expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A) and (b) 'liver-type' 11ß-OHSD mRNA expression (expressed as a percentage of dwarf female levels) in dwarf female rat liver, kidney and hippocampus. $^*p<0.05$ compared to dwarf females (Duncan's Multiple Range test). n=5/group.

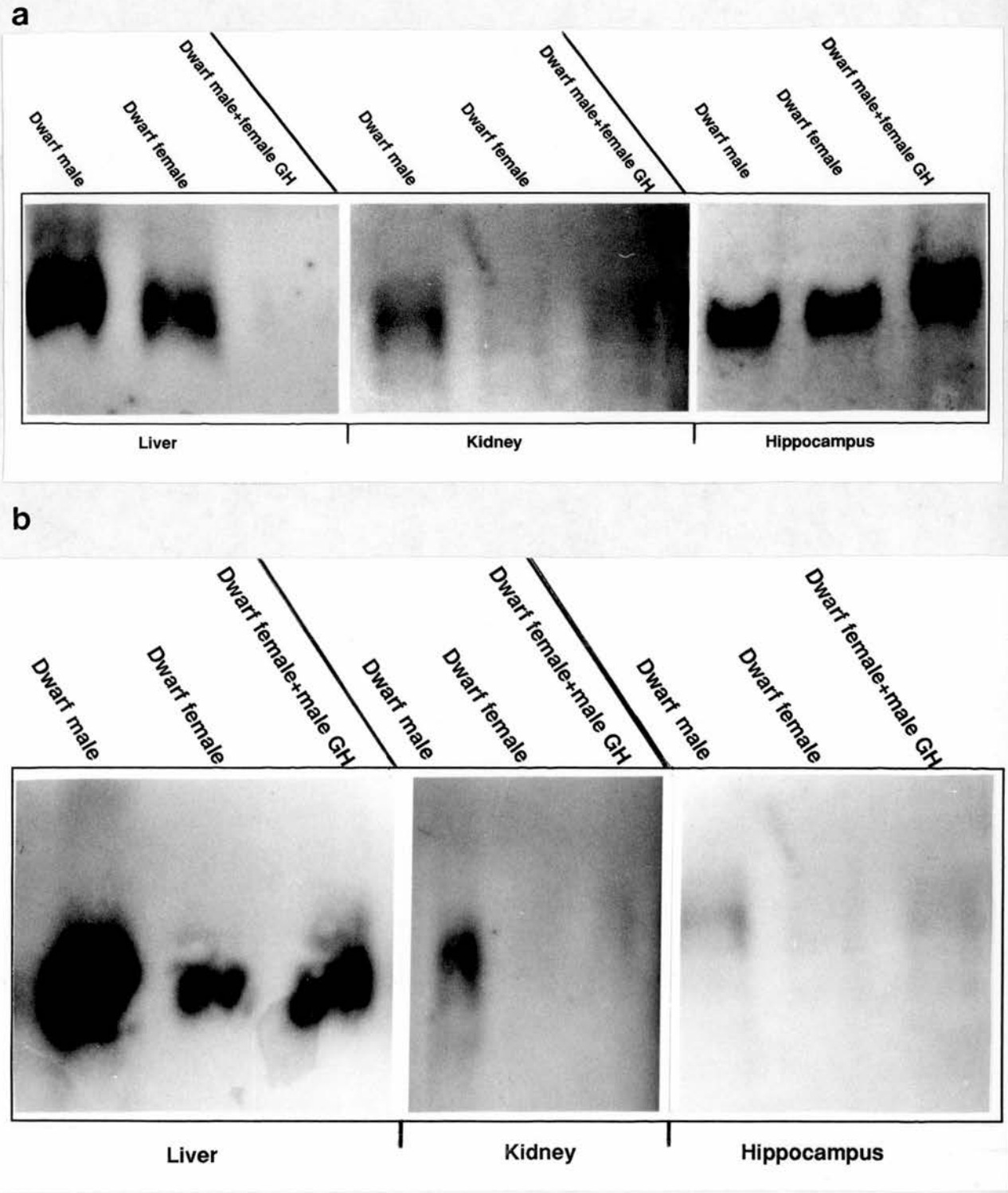



**Figure 5.5:**

Northern blot autoradiographs of 11ß-OHSD mRNA hybridised with 'liver-type' 11ß-OHSD cDNA probes showing the effects of 6d (a) continuous (female pattern) GH in dwarf male rats and (b) pulsatile (male-pattern) GH in dwarf female rats. Each lane represents RNA from a single animal. 11ß-OHSD mRNA expression was quantified by computer densitometry and expressed as a ratio of the optical densities of 11ß-OHSD:7S. The 11ß-OHSD mRNA species in liver and hippocampus is 1.7 kb, while in kidney there are multiple mRNA species of 1.5, 1.6, 1.7 and 1.9 kb.

3h via an automatic injection system) had no effect on hepatic 11ß-OHSD activity in dwarf female animals (Fig. 5.4a). Broadly reflecting changes in hepatic 11ß-OHSD activity, female pattern GH treatment of dwarf male animals led to a decrease (77% fall) in 11ß-OHSD mRNA expression, (Fig. 5.3b, 5.5a), while male pattern GH treatment of dwarf female rats had no effect on hepatic 11ß-OHSD mRNA expression (Fig. 5.4b, 5.5b).

Kidney

Unlike hepatic 11ß-OHSD, 11ß-OHSD activity in kidney does not exhibit a sexually dimorphic pattern of expression in either normal or dwarf rats (Fig. 5.1a). However the levels of 11ß-OHSD activity expressed in dwarf male and female kidney were significantly lower (40% and 34% lower respectively) than those found in parent strain animals (Fig 5.1a). In marked contrast, 'liver-type' 11ß-OHSD mRNA expression in kidney exhibited striking sexual dimorphism in both normal and dwarf rats, with higher levels being expressed in the male animals (77% and 76% higher respectively) (Fig. 5.1b, 5.2).

The lower renal 11ß-OHSD activity in dwarf male and female rats appears to be independent of GH since neither pattern of GH administration, continuous or pulsatile, had any effect on 11ß-OHSD activity in the kidney (Fig. 5.3a, 5.4a). In the same way, neither pattern of GH administration had any effect on 'liver-type' 11ß-OHSD mRNA levels in dwarf male or female kidney (Fig. 5.3b, 5.4b, 5.5a, 5.5b), although there may be a trend towards repression of 'liver-type' 11ß-OHSD mRNA by continuous GH treatment in dwarf male animals (34% decrease compared to dwarf males) (Fig. 5.3b).

Hippocampus

Neither normal nor dwarf rats showed sexually dimorphic patterns of 11ß-OHSD activity or mRNA expression in hippocampus (Fig. 5.1a, 5.1b, 5.2), and neither pattern of GH treatment had any effect on hippocampal 11ß-OHSD (Fig. 5.3a, 5.3b; 5.4a, 5.4b, 5.5a, 5.5b).

### 5.2.2 Effects of Hypophysectomy, GH and Oestradiol on 11ß-OHSD

Liver

To determine whether the smaller sex differences in dwarf rats reflected lower levels of GH, and to establish whether oestradiol had any direct effects on 11ß-OHSD in addition to those of GH, hypophysectomised male Wistar rats treated with GH and/or oestradiol for 6d. To qualify the use of hypophysectomised male rats only, it

was first demonstrated that 11ß-OHSD activity in hypophysectomised male rats was similar to levels of activity expressed both in normal males and hypophysectomised females (Fig. 5.6).

As expected, continuous (female pattern) GH led to a significant decrease in hepatic 11ß-OHSD activity (25% fall) as did oestradiol treatment (26% fall). Combination of oestradiol and GH treatments did not exert an additional effect compared to each hormone alone (Fig. 5.7a). Similarly, the repressive effects of oestradiol and/or GH treatments on hepatic 11ß-OHSD activity following hypophysectomy were reflected by even greater reductions in 11ß-OHSD mRNA expression (Fig. 5.7b, 5.8).

Kidney

Since 11ß-OHSD activity in both normal and dwarf rats in addition to normal Han Wistar rats does not appear to be sexually dimorphic, hypophysectomy would not be expected to effect 11ß-OHSD activity in kidney. However, although levels of 11ß-OHSD activity in normal male and hypophysectomised male liver are similar, hypophysectomy of female rats led to a significant decrease in 11ß-OHSD activity (Fig. 5.6).

Hypophysectomised animals treated with oestradiol for 6d, showed a significant increase in 11ß-OHSD activity (62% increase) compared to hypophysectomy alone (Fig. 5.7a) in agreement with the increase in renal 11ß-OHSD activity observed following oestradiol treatment of gonadectomised male and female rats (Figs. 4.1a, 4.2a). In contrast, continuous GH treatment led to a decrease in renal 11ß-OHSD activity (28% fall), while a combination of oestradiol and GH treatments had no effect on 11ß-OHSD activity in kidney compared to hypophysectomy alone (Fig. 5.7a). In marked contrast to the increase in renal 11ß-OHSD activity following oestradiol treatment of hypophysectomised male rats, 'liver-type' 11ß-OHSD mRNA levels were significantly decreased (74% decrease) (Fig. 5.7b, 5.8). Similarly, although simultaneous oestradiol and GH treatments had no effect on renal 11ß-OHSD activity, they led to a marked decrease in 'liver-type' 11ß-OHSD mRNA expression in kidney (94% fall), compared to hypophysectomy alone (Fig. 5.7b, 5.8). However, continuous GH treatment led to a fall in 'liver-type' 11ß-OHSD mRNA expression, paralleling the changes in enzyme activity (Fig. 5.7b, 5.8). It is interesting to note that the changes in 'liver-type' 11ß-OHSD mRNA expression in kidney broadly reflect the changes in 11ß-OHSD mRNA levels in liver following the same treatments.

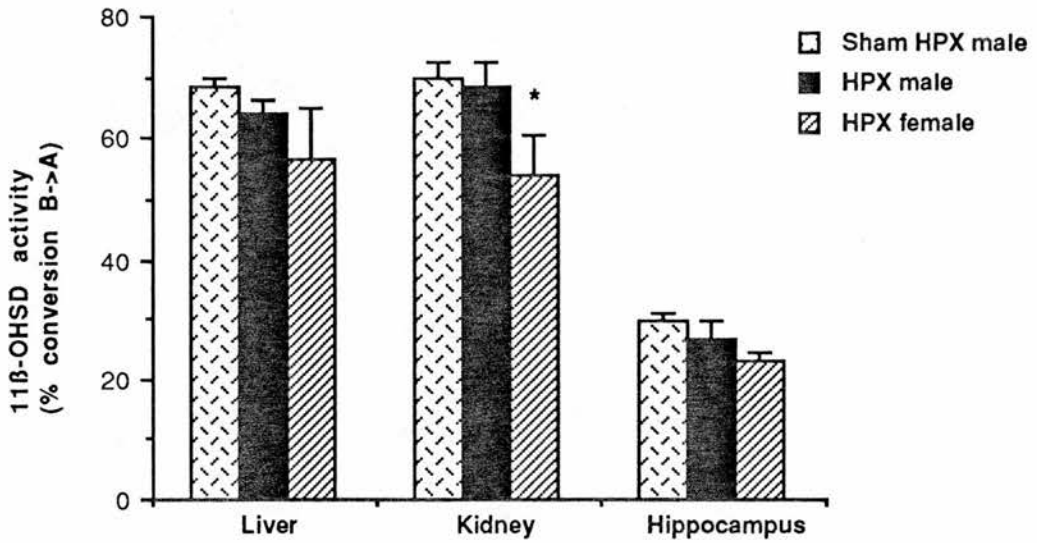



**Figure 5.6:**

11ß-OHSD activity expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A in normal male, hypophysectomised male and hypophysectomised female rat liver, kidney and hippocampus. $^*p<0.05$ compared to normal males (Duncan's Multiple Range test). n=5/group.

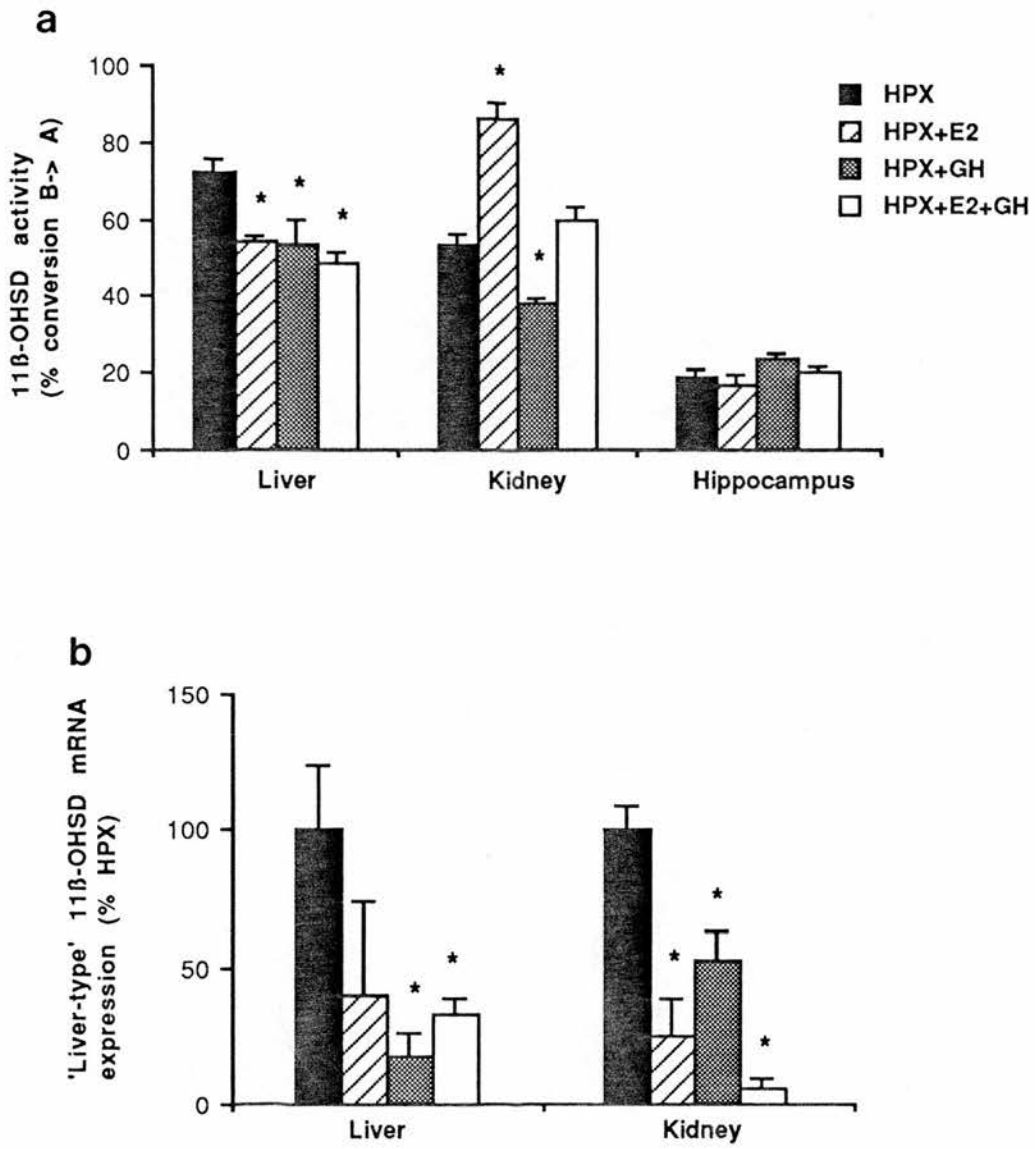



**Figure 5.7:**

Effects of 6d hypophysectomy (HPX) alone or following oestradiol treatment (HPX+E2), GH treatment (HPX+GH) or both (HPX+E2+GH) on (a) 11ß-OHSD activity (expressed as percentage conversion [$^3$H]-B to [$^3$H]-A) and (b) 'liver-type' 11ß-OHSD mRNA expression (expressed as a percentage of hypophysectomy alone) in male rat liver, kidney and hippocampus. $^*p<0.05$ compared to hypophysectomy (Duncan's Multiple Range test). n=5/group.

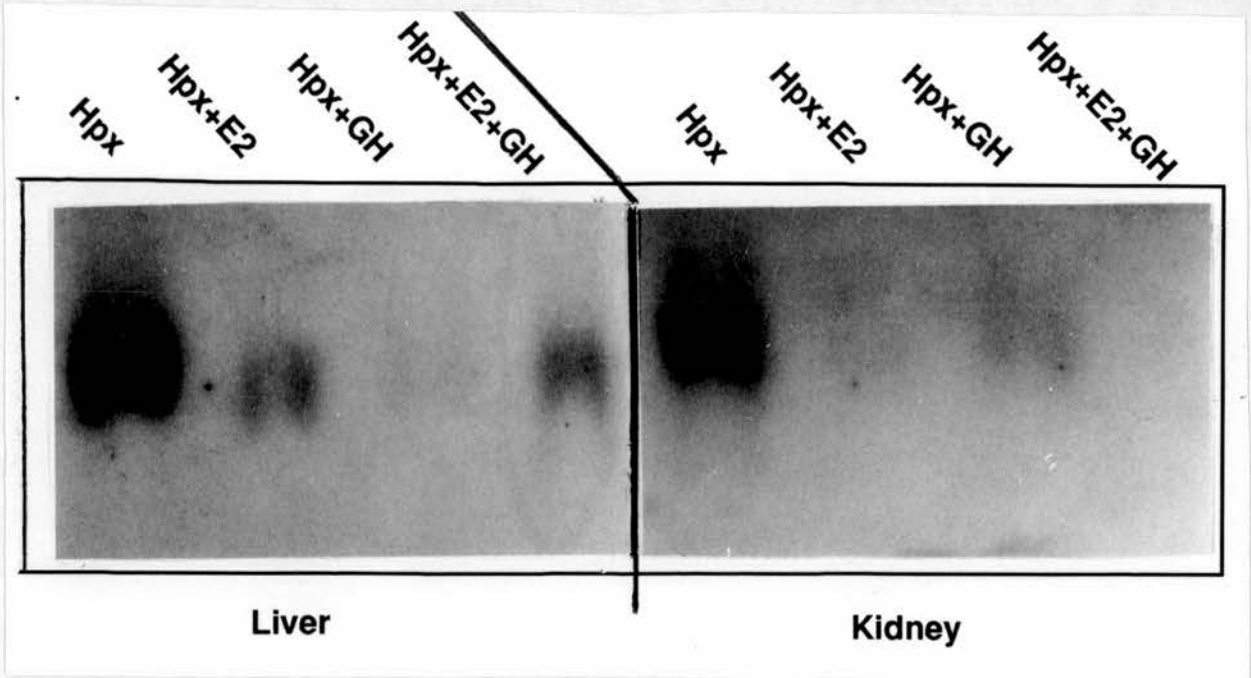



**Figure 5.8:**

Northern blot autoradiograph of 11ß-OHSD mRNA hybridised with $^{32}$P-labelled 'liver-type' 11ß-OHSD cDNA showing the effects of 6d hypophysectomy (HPX) alone, or following oestradiol (HPX+E2), GH (HPX+GH) or oestradiol+GH (HPX+E2+GH) treatments in male rat liver and kidney. Each lane represents RNA from individual animals. 11ß-OHSD mRNA expression was quantified by computer densitometry and expressed as a ratio of the optical densities of 11ß-OHSD:7S. The 11ß-OHSD mRNA species in liver and hippocampus is 1.7 kb, while in kidney there are multiple mRNA species of 1.5, 1.6, 1.7 and 1.9 kb.

Hippocampus

Hypophysectomised male and female rats exhibited similar levels of hippocampal 11ß-OHSD activity to normal males (Fig. 5.6).

Hypophysectomy followed by oestradiol and growth hormone treatments either alone or in combination, had no effect on 11ß-OHSD activity (Fig. 5.7a).

## 5.3 Discussion

In Chapter 4, I demonstrated that hepatic 11ß-OHSD was expressed at lower levels in female Han Wistar rats due to oestradiol repression of 11ß-OHSD in female liver. This sexual dimorphism in hepatic 11ß-OHSD activity has now also been demonstrated in a second normal (AS) strain of rat. These effects are likely to be mediated at least in part, at the level of transcription, as reflected by the sexual dimorphism of hepatic 11ß-OHSD mRNA expression. Dwarf animals also exhibit sexual dimorphism of 11ß-OHSD, but to a lesser extent than parent strain rats indicating that this may be related to GH, since this dwarf strain is not deficient in sex steroids or any other pituitary-dependent hormones apart from GH (Charlton et al., 1988). This hypothesis is supported by the sex-specific patterns of GH administration in dwarf rats which indicate that female-specific GH secretion may be responsible for repression of 11ß-OHSD activity and mRNA expression in female liver. Since dwarf female rats expressed significantly lower levels of 11ß-OHSD than dwarf males, it could be argued that repression of 11ß-OHSD by continuous GH treatment in dwarf males would be more easily observed than putative repression resulting from pulsatile GH treatment in dwarf female animals. However, both sexes of dwarf rats expressed significantly higher levels of hepatic 11ß-OHSD activity than normal females, and these levels were not significantly different from normal males, indicating that there should be enough scope to observe any effects of hepatic 11ß-OHSD by pulsatile GH. Interestingly, it has recently been demonstrated that although pituitary growth hormone production is much less in dwarf animals, the sex-specific secretory profiles are maintained (Legraverend et al., 1992) which may explain why sexually dimorphic expression of hepatic 11ß-OHSD is still evident in dwarf rats, but to a much lesser extent than in normal animals. This is further supported by recent work on members of the cytochrome $P_{450}$ 2C gene family and other steroid metabolising enzymes which exhibit sexually dimorphic expression mediated by the physiological patterns of GH secretion (Gustafsson et al., 1983). In dwarf rats where plasma growth hormone levels are 10 to 50-fold lower than normal, the sexually dimorphic expression of the cytochrome $P_{450}$ 2C gene family (Bullock et al., 1991; Legraverend et al., 1992) and 5α-reductase (Bullock et al., 1991) is

maintained indicating that even low levels of GH are sufficient to maintain sexually differentiated expression of many hepatic steroid metabolising enzymes including 11ß-OHSD.

To address the possible effects of residual GH in the dwarf rats, and determine any effects of oestradiol in the absence of GH, hypophysectomised male rats were treated with continuous GH and/or oestradiol for 6d. Supporting the results in dwarf animals, GH treatment repressed 11ß-OHSD activity, and particularly mRNA expression in rat liver. Interestingly, 6d oestradiol treatment following hypophysectomy also led to significant repression of hepatic 11ß-OHSD, which could not be due to GH, and may therefore be a direct effect of oestradiol. However it should be noted that the effects of oestradiol were investigated in male rat liver, and from the results in Chapter 5, it is evident that the dose of oestradiol administered may not result in plasma oestrogen levels similar to those found in normal females as previously reported (Painson et al., 1992), and therefore pharmacological effects of oestradiol on male hepatic 11ß-OHSD cannot be ruled out. Thus although oestradiol does repress 11ß-OHSD in these experiments, whether this is entirely a pharmacological effect cannot be determined from this study. It is also interesting to note that Lax et al (1978) did not find an effect of oestradiol or testosterone on male rat liver 11ß-OHSD activity following hypophysectomy which may reflect differential effects of oestradiol dosage. Whether oestradiol acts directly or indirectly on hepatic 11ß-OHSD remains undetermined. One experimental point which is prominent from this study, is that following any hormone treatment, effects on 11ß-OHSD mRNA expression in liver (and kidney) appear to be magnified with respect to the effects on 11ß-OHSD activity. Since this is also the case when simply observing 11ß-OHSD in males and females which have not undergone any treatment, this suggests that northern analysis may be a more sensitive method for detecting differences in 11ß-OHSD expression. However in addition, following hormonal treatments, it may be a reflection of initial suppression of transcription, and/or the greater stability of existing 11ß-OHSD protein than mRNA, and if the treatment period was extended, the levels of 11ß-OHSD activity may decrease in line with mRNA expression.

The mechanism(s) of regulation of 11ß-OHSD by GH are as yet unknown. The rat cytochrome P450 2C11, 2C12 and 2C13 genes encode a testosterone 16α-hydroxylase (Cheng & Schenkman, 1982), a steroid sulphate 15ß-hydroxylase (MacGeoch et al., 1984) and a testosterone 6ß-hydroxylase (Ryan et al., 1984) respectively. These are sex specific forms of P450 that are developmentally regulated at a pretranscriptional level by the sexually dimorphic pattern of GH secretion (MacGeoch et al., 1987; McClellan et al., 1989; Strom et al., 1988). In hepatocytes

isolated from male rats, continuous (female pattern) GH added to hormone free medium is sufficient to induce expression of the female-specific 2C12 mRNA and protein within 4h (Guzelian et al., 1988; Tollet et al., 1990) and repress expression of the male-specific 2C11 and 2C13 mRNAs (Legraverend et al., 1992). This indicates that GH acts directly on the hepatocyte to regulate sex-specific expression of cytochrome P450 2C11, 2C12 and 2C13 genes. Whether this mechanism is also responsible for the sexual dimorphism of 11ß-OHSD expression remains to be determined.

The physiological implications of lower hepatic 11ß-OHSD activity and mRNA expression are not known. It is believed that the prominent direction of 11ß-OHSD in liver is in the reductase direction thus leading to accumulation of active glucocorticoids (Bush et al., 1969). If, due to lower levels of hepatic 11ß-OHSD, there were lower levels of active glucocorticoids achieved in female liver, this may explain differences in glucocorticoid-sensitive hepatic genes, such as those involved in growth and drug and alcohol metabolism (Edenberg & Brown, 1992), as well as perhaps having an involvement in the increased incidence of glucocorticoid-responsive inflammatory disorders in human female liver.

Regulation of the 'liver-type' 11ß-OHSD mRNA in kidney largely parallels the changes observed in the liver. 'Liver-type' 11ß-OHSD mRNA expression in kidney is higher in normal and dwarf male rats than in normal and dwarf female rats respectively. Similarly in parallel with the liver, continuous (female pattern) but not pulsatile (male pattern) GH replacement led to a decrease in 'liver-type' 11ß-OHSD mRNA expression in kidney, although this did not reach significance. In marked contrast, 11ß-OHSD activity in kidney was not sexually dimorphic in normal or dwarf animals, perhaps indicating the necessity for protection of renal mineralocorticoid receptors from exposure to glucocorticoid exposure at all times. However, renal 11ß-OHSD activity was lower in dwarf rats and independent of GH patterns. Collectively, the repression of all renal transcripts of the 'liver-type' 11ß-OHSD gene (Agarwal et al., 1989) suggest that this isoform of 11ß-OHSD is unlikely to be responsible for all of the substantial renal activity observed, which instead are likely to result from the product(s) of an alternative 11ß-OHSD gene(s) which is not affected by differential patterns of GH secretion. The results of hormone replacement following hypophysectomy emphasise the complexities of renal 11ß-OHSD activity. 6d oestradiol treatment led to marked repression of the 'liver-type' 11ß-OHSD mRNA expression, despite having the opposite effect on total renal 11ß-OHSD activity presumably reflecting induction of an additional 11ß-OHSD gene product by oestradiol. Continuous GH treatment in contrast led to a slight decrease in 11ß-OHSD activity, and more marked decrease in 11ß-OHSD mRNA expression, perhaps

reflecting a more subtle regulation of the additional gene product by GH, or more likely in light of the effects on 11ß-OHSD in liver, reflecting regulation solely of the 'liver-type' 11ß-OHSD isoform by GH.

Although 11ß-OHSD activity and in dwarf rat kidney does not appear to be sexually dimorphic, 11ß-OHSD activity in dwarf rat kidney was significantly lower than in normal animals. The implications of this for dwarf rats in terms of renal mineralocorticoid function, are not known. However it may be possible to determine the effect of lower 11ß-OHSD actvitiy in dwarf kidney in terms of protection of renal mineralocorticoid receptors from glucocorticoid exposure by comparing the *in vivo* binding of [$^3$H] corticosterone in dwarf rat kidney and normal rat kidney.

Hippocampal 11ß-OHSD activity and mRNA expression did not exhibit sexual dimorphism in normal or dwarf animals, and were not affected by either pattern of GH administration. This could be easily explained if the effects of sex steroids were entirely mediated through modification of patterns of GH secretion, since GH like may other peptide hormones does not cross the blood brain barrier and would therefore not be expected to affect hippocampal 11ß-OHSD. In agreement with this, hypophysectomy followed by 6d GH or oestradiol treatments did not affect hippocampal 11ß-OHSD activity. However hippocampal and hepatic 11ß-OHSD predominantly utilise the same promoter and produce mRNA transcripts of the same size (Low et al., 1993; Smith et al., 1993), yet following hypophysectomy hepatic 11ß-OHSD was repressed by oestradiol treatment while the hippocampal enzyme remained unaffected. It is likely therefore that hippocampal and hepatic 11ß-OHSD are differentially regulated by as yet undetermined factors. For example subcellular compartmentalisation of 11ß-OHSD in liver and hippocampus may not be the same, and in addition it is likely that tissue-specific transcription factors regulate expression of 11ß-OHSD in a tissue-specific manner (Williams et al, 1993), and that the regulatory factors governing expression of 11ß-OHSD in liver and hippocampus may differ. This contention is supported by the presence of functional oestrogen receptors in hippocampus (Bettini et al., 1992) and therefore it is possible that oestrogen receptors are acting in the liver through an effect on another transcription factor not present in hippocampus to differentially regulate 11ß-OHSD, but this mechanism(s) has yet to be addressed.

In conclusion, the work in this chapter demonstrates that the sexual dimorphism of hepatic 11ß-OHSD may be mediated in part by sex-specific patterns of GH secretion. However expression of 11ß-OHSD in liver can also be regulated through an action of oestradiol, not dependent on GH. Hippocampal 11ß-OHSD is not regulated by GH, furthermore it does not appear to be regulated by oestradiol in

contrast to liver and kidney, suggesting as yet undetermined tissue-specific differences in the regulation of hippocampal and hepatic 11ß-OHSD. Finally, although regulation of 'liver-type' 11ß-OHSD mRNA in rat kidney largely parallels that of the liver, regulation of renal 11ß-OHSD activity remains complex due to the involvement of an additional isoform of 11ß-OHSD in rat kidney contributing to the total enzyme activity measured in this tissue.

# CHAPTER 6

# REGULATION OF 11ß-OHSD IN INTACT MAMMALIAN CELLS

## 6.1 Introduction

Although 11ß-OHSD activity is expressed in many tissues (Monder & Shackleton, 1984), its physiological role is only really understood in kidney where the enzyme protects MR from exposure to glucocorticoids. Antibodies raised against purified rat liver 11ß-OHSD (Lakshmi & Monder, 1988) failed to co-localise renal 11ß-OHSD and MR leading to the suggestion that 11ß-OHSD detected in proximal tubules may regulate glucocorticoid access to MR in distal convoluted tubules and cortical collecting ducts by a paracrine mechanism (Edwards et al., 1988; Rundle et al., 1989). However using the cDNA encoding 11ß-OHSD which was cloned from a rat liver cDNA library (Agarwal et al., 1989), 11ß-OHSD mRNA expression was found to be high in distal as well as proximal epithelia (Yau et al., 1991).

When 11ß-OHSD was purified, it was found to express only dehydrogenase activity suggesting that the reductase component of 11ß-OHSD may be a distinct enzyme, and supporting the proposed role of 11ß-OHSD as a protective mechanism (Lakshmi & Monder, 1988). However when the cloned 11ß-OHSD cDNA was expressed in human osteosarcoma cells at relatively high levels using recombinant vaccinia virus, 11ß-OHSD was found to exhibit approximately equal amounts of dehydrogenase and reductase activities, with µM affinity for both corticosterone and 11-dehydrocorticosterone (Agarwal et al., 1990). Furthermore, endogenous glucocorticoids circulate 'free' in low nM concentrations, and mineralocorticoid receptors have a Kd for glucocorticoids which is ~0.5nM (Arriza et al., 1987; Krozowski & Funder, 1983) making it unlikely that the 'liver-type' 11ß-OHSD could effectively compete for glucocorticoid binding. Therefore it was predicted that 11ß-OHSD would not be able to protect MR and GR from exposure to low (physiological) concentrations of glucocorticoids, but would adequately inactivate high levels of glucocorticoids.

Recently, a completely different cell culture system; the toad bladder cell line (TBM 18-23) has been used to look at the direction of 11ß-OHSD activity (Dupperex et al., 1993). Stable transfection of TBM 18-23 cells with the entire coding region of

rat liver 11ß-OHSD, resulted in expression of 11ß-reductase activity suggesting that in this intact cell system, rat liver 11ß-OHSD is capable of activating inert glucocorticoids. However dehydrogenase activity of 11ß-OHSD could not be assessed in this amphibian cell line since it was demonstratied that dexamethasone treatment of TBM 18-23 cells increased endogenous 11ß-OHSD activity in the dehydrogenase direction. Therefore when transfected TBM 18-23 cells were treated with dexamethasone, the resulting increase in 11ß-dehydrogenase activity could not be contributed to the transfected 11ß-OHSD plasmid.

'Liver-type' 11ß-OHSD mRNA expression is co-localised with MR mRNA in hippocampus (section 3.2.4, Fig. 3.6a) and may in addition, regulate access of glucocorticoids to GR (Moisan et al., 1990b; Teelucksingh et al., 1990; Fuller & Verity, 1990). Indeed 11ß-OHSD and GR mRNAs have been demonstrated in the same tissues (Whorwood et al., 1992; section 3.2.4, Fig. 3.6b) suggesting additional roles for 11ß-OHSD in regulating glucocorticoid access to GR in glucocorticoid responsive tissues (eg liver) as well as to MR in tissues which express MR but respond to physiological glucocorticoids (eg hippocampus). Therefore in order to test the hypothesis that 'liver-type' 11ß-OHSD may regulate glucocorticoid access to GR as either a dehydrogenase or reductase, I transfected the rat 'liver-type' 11ß-OHSD cDNA into mammalian cell lines (COS-7, CV-1) and assessed the ability of 11ß-OHSD in intact cells to metabolise glucocorticoids and correlated this with 11ß-dehydrogenase activity in homogenates of the same cells. In addition, I looked at a molecular marker for glucocorticoid activity, namely the ability of corticosterone and 11-dehydrocorticosterone to regulate MMTV-luciferase expression in the presence and absence of 11ß-OHSD.

## 6.2 Results

pSL1 was created by cloning the 1.2kb EcoR1/Sst1 fragment of rat liver 11ß-OHSD (encoding the entire open reading frame) into pJ3 (Morgenstern & Land, 1990) placing expression under the SV40 promoter (described in section 2.1.2.3). 0.1-10µg of pSL1 was transiently transfected into COS-7 cells, and 11ß-OHSD activity in both dehydrogenase and reductase directions measured in intact cells over 24h by monitoring the conversion of [$^3$H]-corticosterone ([$^3$H]-B) to [$^3$H]-11-dehydrocorticosterone ([$^3$H]-A) or [$^3$H]-A to [$^3$H]-B in the cell medium (as detailed in section 2.6.2.1). 11ß-OHSD activity in homogenates of the same cells was also measured (as described in section 2.6.2.3), for comparison with activities found in the medium.

### 6.2.1 Efficiency of Transfection of COS-7 Cells

COS-7 cells were transiently transfected with pKC275, a plasmid constructed by Karen Chapman, encoding ß-galactosidase expression. Cells were subsequently fixed with 4% paraformaldehyde and stained with X-Gal (as described in section 2.6.4) to determine the number of cells typically transfected. In an average of six cell culture dishes transfected, the percentage of cells positively stained for ß-galactosidase in this way was 45±1.5% (Fig. 6.1). COS-7 cells transfected with the control plasmid pGEM3, showed no ß-galactosidase staining by this method.

### 6.2.2 Rat 'Liver-Type' 11ß-OHSD in COS-7 Cells

Intact mock transfected COS-7 cells contained little 11ß-OHSD activity in either dehydrogenase or reductase directions. In intact cells transfected with 0.1-10µg pSL1, no 11ß-dehydrogenase activity was detectable above mock transfected levels at 24h (Fig. 6.2a). In contrast, 11ß-reductase activity measured by conversion of [$^3$H]-A to [$^3$H]-B in intact COS-7 cells was readily apparent in cells transfected with 10µg pSL1 at 24h, and apparent to a lesser extent in cells transfected with 1µg or 3µg of pSL1 (Fig. 6.3a).

To correlate metabolism of glucocorticoids seen in cell medium with 11ß-OHSD activity present in cell homogenates, 11ß-dehydrogenase activity was measured in homogenates of the same cells. In both cases, conversion of [$^3$H]-B to [$^3$H]-A was apparent in cell homogenates transfected with 1µg pSL1, and was maximal in homogenates from cells transfected with 10µg pSL1 (Figs. 6.2b; 6.3b). In both cases therefore, similar levels of *in vitro* 11ß-OHSD activity were obtained indicating that similar levels of expression of transfected plasmid result in 11ß-reductase (Fig. 6.3a), but not 11ß-dehydrogenase (Fig. 6.2a) activity in intact cells. The lack of dehydrogenase activity in intact cells cannot therefore be explained by lack of expression of transfected pSL1.

The time course of 11ß-dehydrogenase and 11ß-reductase activities in the intact cells was also measured. As expected no 11ß-dehydrogenase activity was detected at any of the time points measured even at maximal levels of transfected of pSL1 (Fig. 6.4a). However, 11ß-reductase activity in cells transfected with 10µg pSL1 was apparent within 4h of addition of [$^3$H]-A to the medium, and by 24h, 11ß-reductase activity was detectable in intact cells transfected with 0.3µg, 1µg and 3µg pSL1 (Fig. 6.4b).

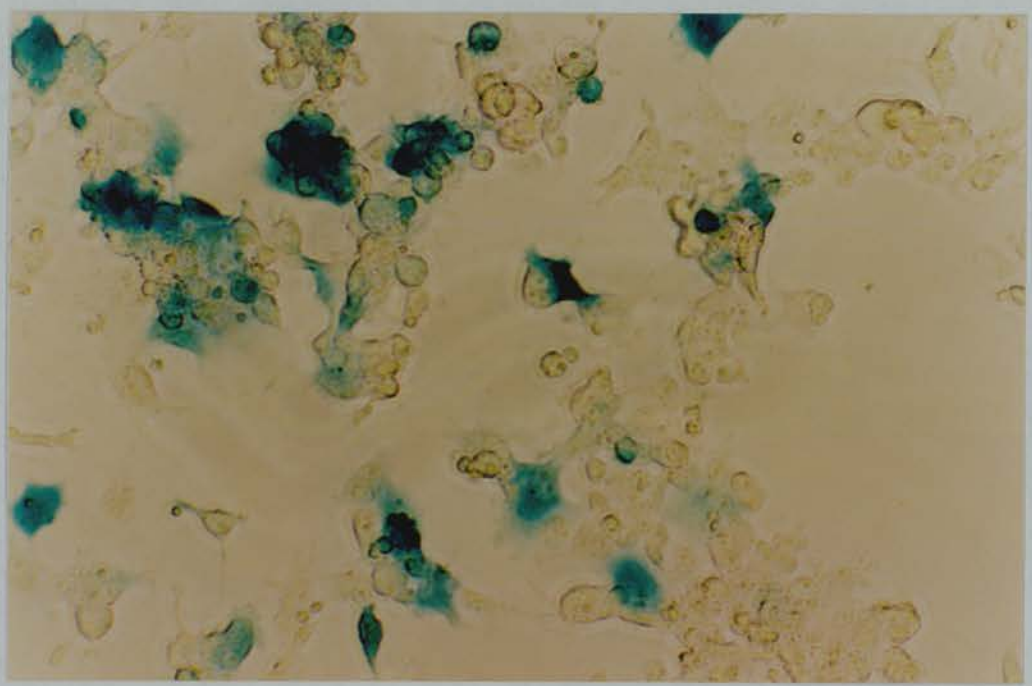


**Figure 6.1:**
X-Gal staining in situ as a measure of the efficiency of transfection. Cos-7 cells were transfected with pKC275 (plasmid encoding ß-galactosidase), fixed in paraformaldehyde and incubated in a solution containing X-Gal. This is a representative experiment which was repeated three times.

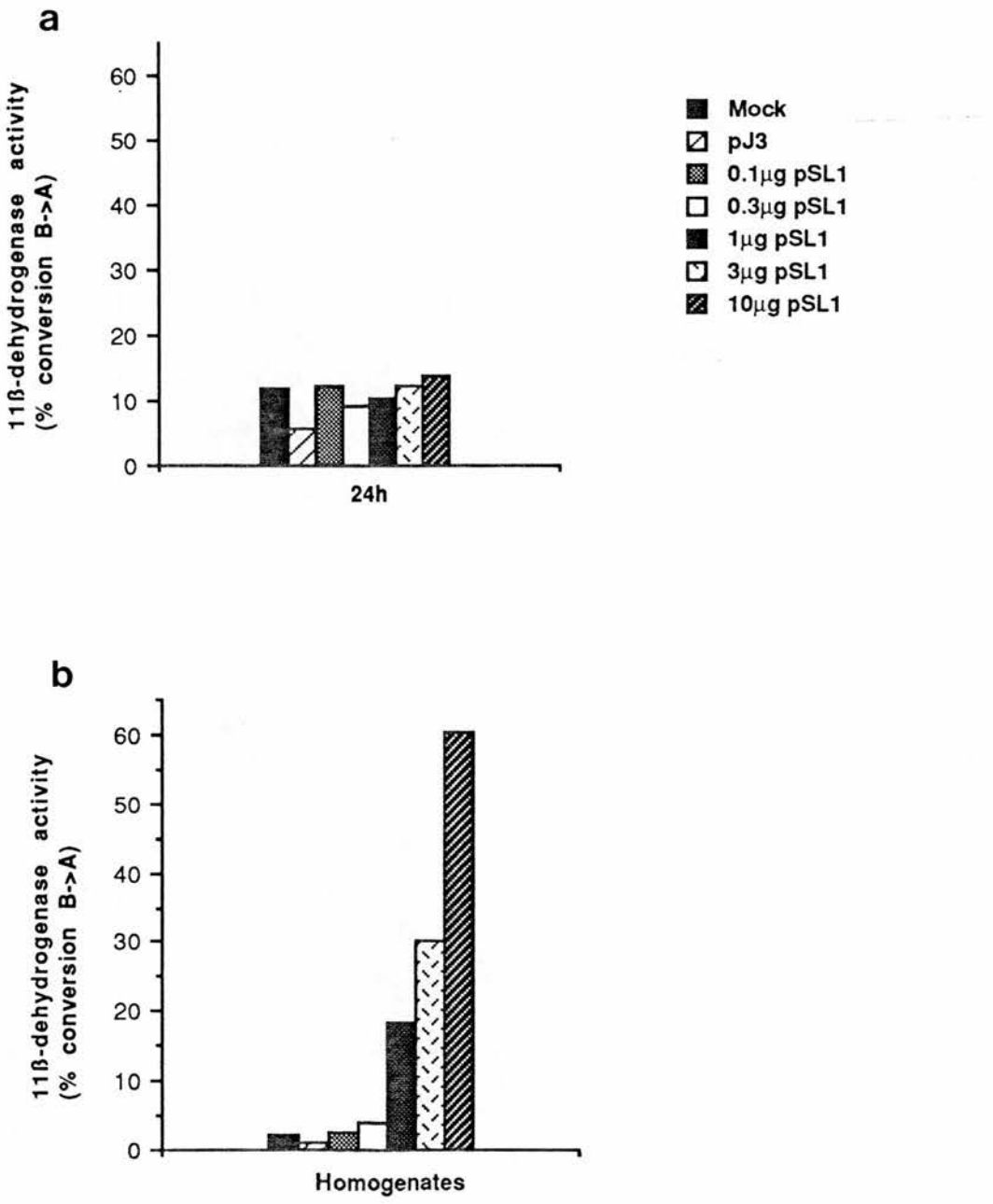



**Figure 6.2:**

The ability of 'liver-type' 11ß-OHSD cDNA to act as a dehydrogenase in transiently transfected COS-7 cells. (a) 11ß-dehydrogenase activity in intact cells measured and expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A in the cell medium. (b) 11ß-dehydrogenase activity in homogenates of the same cells, measured and expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A. Data are from a representative experiment which was repeated three times. All values are expressed as means of duplicate measurements.

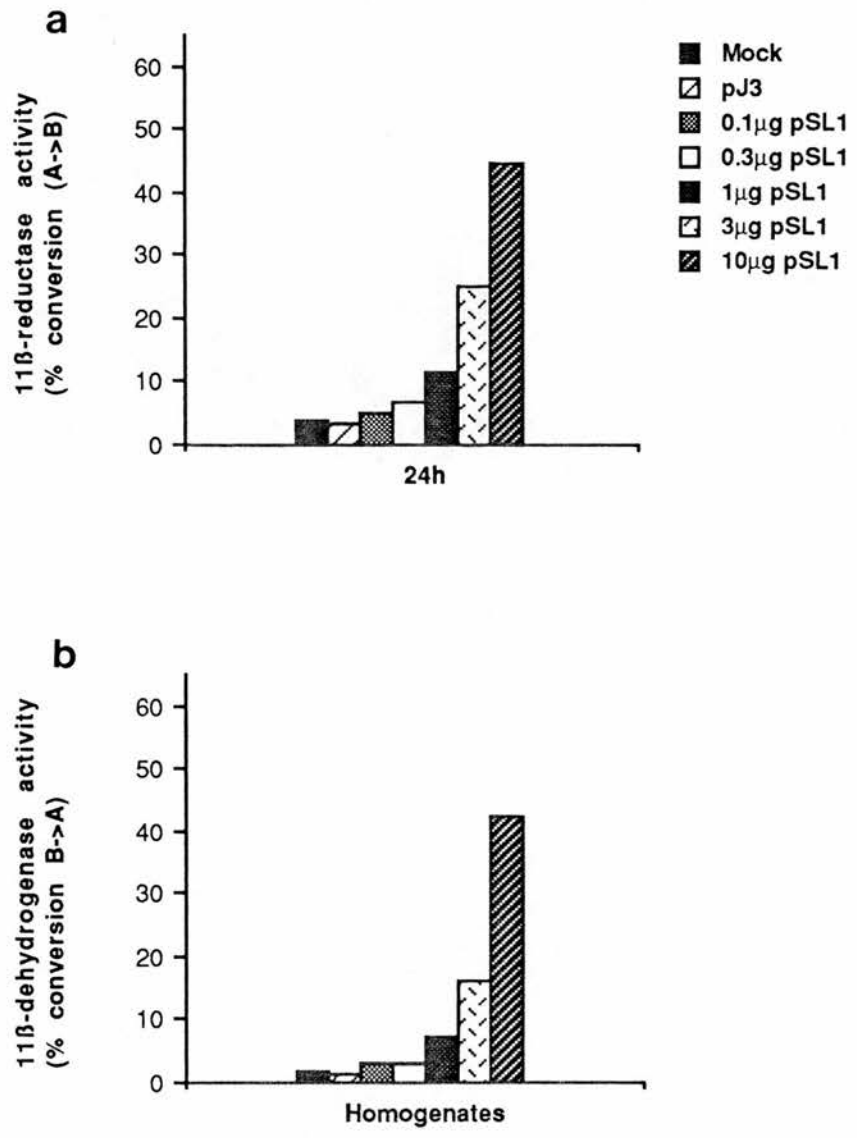



**Figure 6.3:**

The ability of 'liver-type' 11ß-OHSD cDNA to act as a reductase in transiently transfected COS-7 cells. (a) 11ß-reductase activity in intact cells measured and expressed as percentage conversion of [$^3$H]-A to [$^3$H]-B in the cell medium. (b) 11ß-dehydrogenase activity in homogenates of the same cells measured and expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A. Data are from a representative experiment which was repeated three times. All values are expressed as means of duplicate measurements.

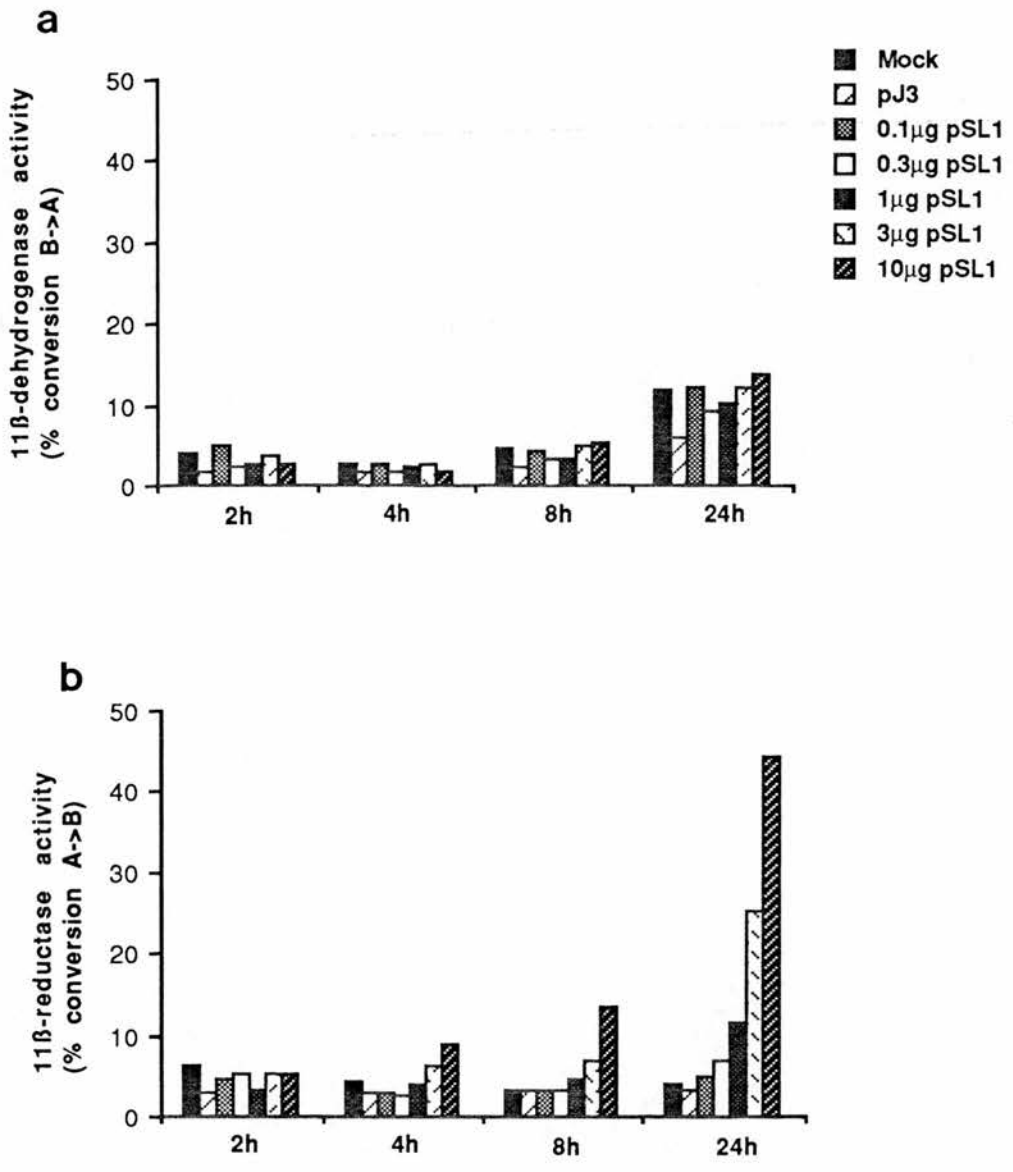



**Figure 6.4:**

Time couse of expression of 11ß-dehydrogenase and 11ß-reductase activities of rat 'liver-type' 11ß-OHSD cDNA transfected into COS-7 cells. (a) Development of 11ß-dehydrogenase activity measured and expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A in the cell medium. (b) Development of 11ß-reductase activity measured and expressed as percentage conversion of [$^3$H]-A to [$^3$H]-B in the cell medium. This is a representative experiment which was repeated twice. All values are means of duplicate measurements.

### 6.2.3 Expression of Rat 'Liver-Type' 11ß-OHSD in CV-1 Cells

In order to determine that predominant 11ß-OHSD reductase activity in COS-7 cells was not unique to the particular cell line, similar experiments were carried out in CV-1 cells which do not contain MR or GR (Giguere et al., 1986; Arriza et al., 1987) or detectable 11ß-OHSD activity or mRNA expression (Low et al., unpublished observations).

In contrast to COS-7 cells, pSL1 transfected CV-1 cells did show a small amount of dehydrogenase activity in intact cells which was maximal/optimal at 3µg (Fig. 6.5a). Reductase activity in intact cells was not measured. Despite the very low level of dehydrogenase activity in intact cells, enzyme activity in cell homogenates was readily detectable, and was optimal/maximal in homogenates of CV-1 cells previously transfected 3µg pSL1 (Fig. 6.5b). Thus although 3µg pSL1 produced maximal levels of 11ß-dehydrogenase activity in intact and homogenised CV-1 cells, higher 11ß-dehydrogenase activity in COS-7 cell homogenates and 11ß-reductase activity in intact COS-7 cells was observed following transfection with 10µg pSL1.

### 6.2.4 Influence of 11ß-OHSD on Activity of a Glucocorticoid Responsive Reporter Gene

To investigate the ability of 11ß-OHSD to regulate access of glucocorticoids to GR *in vivo*, COS-7 cells were transfected with a human GR expression plasmid (Giguere et al., 1986) and a glucocorticoid responsive reporter gene (MMTV-LTR luciferase) in the presence or absence of 11ß-OHSD (pSL1). Following transfection, corticosterone and 11-dehydrocorticosterone were added to the medium as described in section 2.6.5.

Transfection of MMTV-LTR luciferase alone in the absence of GR or steroid resulted in 7.5-fold induction of luciferase activity higher than background (Table 6.1). Addition of GR alone, in the absence of added steroid resulted in a 16-fold increase in expression of MMTV-LTR luciferase. This was further increased to a 36-fold increase in the presence of 10nM dexamethasone. Addition of pJ3 or pSL1 also led to an increase in GR induction of MMTV-LTR luciferase in the absence of added steroid (34-fold and 34-fold increase respectively) or in the presence of 10nM dexamethasone (144-fold and 143-fold induction respectively). At concentrations of B below 10nM, MMTV-LTR luciferase activity in the presence of GR was similar to MMTV-LTR luciferase activity measured in the absence of steroids (Table 6.1). However, addition of 10nM to 1µM B resulted in increased expression of MMTV-

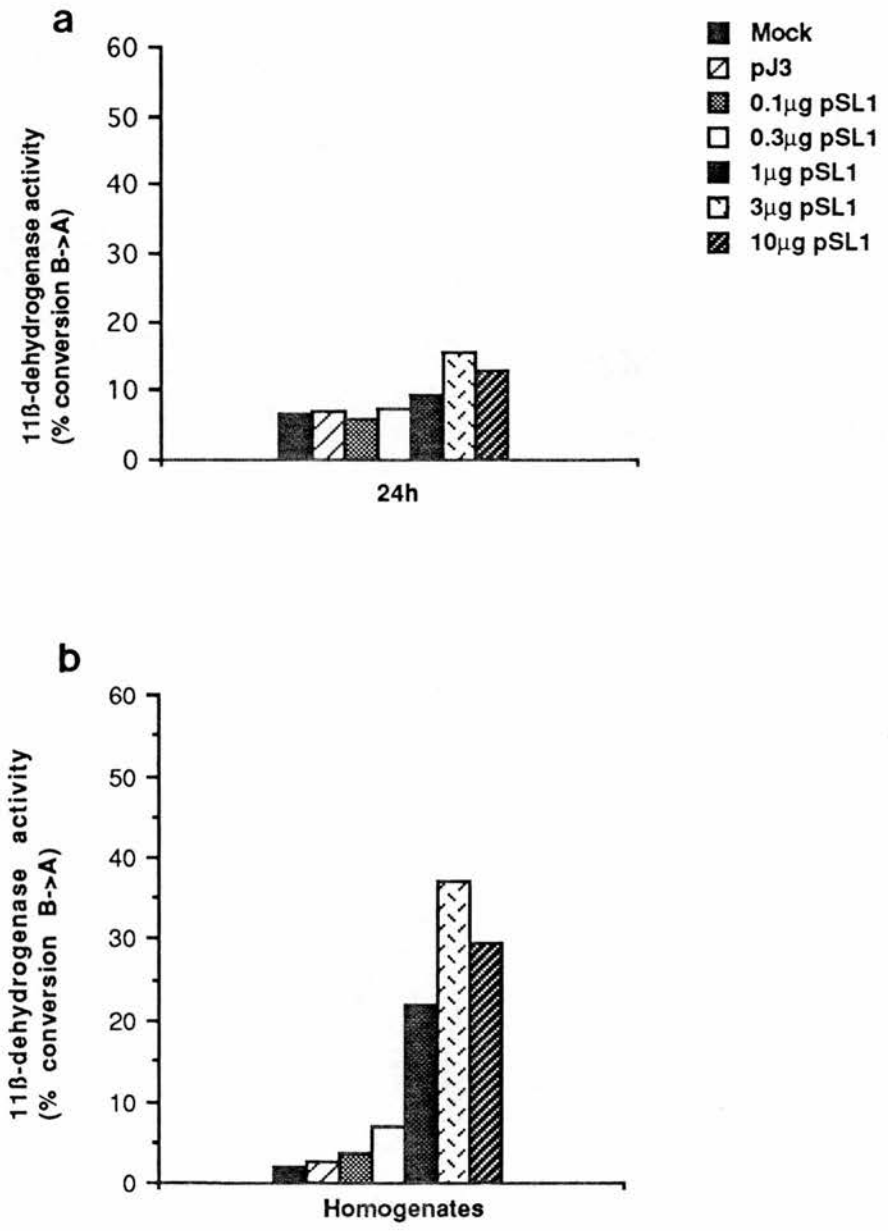



**Figure 6.5:**

The ability of 'liver-type' 11ß-OHSD cDNA to act as a dehydrogenase in transiently transfected CV-1 cells. (a) 11ß-dehydrogenase activity in intact cells measured and expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A in the cell medium. (b) 11ß-dehydrogenase activity in homogenates of the same cells measured and expressed as percentage conversion of [$^3$H]-B to [$^3$H]-A. Data are from a representative experiment which was repeated three times. All values are means of duplicate measurements.

| | Fold Induction of MMTV-LTR luciferase (over mock transfected cells) in the presence of | | | |
|---|---|---|---|---|
| | 0 | GR | GR + pJ3 | GR + pSL1 |
| No steroid | 7.5 | 16 | 34 | 34 |
| Dex 10nM | 3 | 36 | 144 | 143 |
| B 0.5nM | | | | 39 |
| 1.0nM | | | | 49 |
| 3.0nM | | | 38 | 48 |
| 10nM | | | | 54 |
| 100nM | | | | 79 |
| 1000nM | | | 71 | 114 |
| A 0.5nM | | | | 44 |
| 1.0nM | | | | 34 |
| 3.0nM | | | 38 | 43 |
| 10nM | | | | 47 |
| 100nM | | | | 42 |
| 1000nM | | | 33 | 71 |

**Table 6.1:**

The ability of corticosterone and 11-dehydrocorticosterone to regulate glucocorticoid receptor (GR) activation of the glucocorticoid responsive gene MMTV-luciferase in the presence (pSL1) or absence (pJ3) of 11ß-OHSD in COS-7 cells. Data are expressed as fold induction of cells transfected with GR+10µg pJ3 in the absence of steroid. These results are representative; each experiments was repeated three times. Dex=dexamethasone, B=corticosterone, A=11-dehydrocorticosterone.

LTR luciferase, although the increase was not as large as expected perhaps due to the unexpectedly high induction of the reporter gene by GR in the absence of steroid masking low level increases in expression (Table 6.1; 6.2). 11-dehydrocorticosterone however, which is not able to bind to or activate mineralocorticoid or glucocorticoid receptors, was found to induce MMTV-LTR luciferase only in the presence of pSL1, indicating that the 'liver-type' 11ß-OHSD cDNA is predominantly acting in the reductase direction and at 1µM A, can produce sufficient B to induce MMTV-LTR luciferase activity through activation of GR (Table 6.1; 6.2).

| | Fold induction of MMTV-LTR luciferase activity in the presence of | |
|---|---|---|
| | 10µg pJ3 | 10µg pSL1 |
| No steroid | 1 | 1 |
| Dex 10nM | 4.2 | 4.2 |
| B 1µM | 2.1 | 3.4 |
| A 1µM | 1.1 | 2.1 |

**Table 6.2:**
The ability of corticosterone and 11-dehydrocorticosterone to regulate glucocorticoid receptor (GR) activation of the glucocorticoid responsive gene MMTV-luciferase in the presence (pSL1) or absence (pJ3) of 11ß-OHSD in COS-7 cells. Data are expressed as fold induction over cells transfected with 10µg pJ3 in the absence of steroid. These results are representative; each experiments was repeated three times. Dex=dexamethasone, B=corticosterone, A=11-dehydrocorticosterone.

## 6.3 Discussion

We and others have proposed, based on co-localisation studies, that 11ß-OHSD plays a role *in vivo* to protect corticosteroid receptors from exposure to glucocorticoids. Others have demonstrated dehydrogenase and reductase activities in mammalian cells (encoded by the 'liver-type' 11ß-OHSD) in approximately equal proportions *in vitro* (Agarwal et al., 1990) and *in vivo* (Agarwal et al., 1989). In addition, *in vivo* 11ß-reductase activity was reported in intact amphibian cells (it was not possible due to experimental design to measure dehydrogenase activity in these cells) (Dupperex et al., 1993). Therefore in amphibian and mammalian cells, it has

been shown that 'liver-type' 11ß-OHSD can encode 11ß-reductase activity. In this chapter I have shown that at least in some mammalian cell types, 11ß-reductase activity predominates in intact cells (despite high levels of dehydrogenase activity in homogenates) precluding a role for the 'liver-type' 11ß-OHSD in inactivating steroids. Furthermore I have shown that co-expression of 11ß-OHSD with GR permits activation of a glucocorticoid-sensitive reporter gene by the otherwise inactive glucocorticoid, 11-dehydrocorticosterone.

In these experiments GR alone, in the absence of added steroid, activated MMTV-LTR luciferase activity. This was unexpected as all serum used in these experiments was charcoal stripped to remove endogenous steroids. The stripping procedure was 99.8% effective (assessed by removal of added [$^3$H]-B; data not shown). Therefore the effect of GR on the reporter gene is unlikely to be due to glucocorticoids in the cell medium. The activation of MMTV-LTR luciferase by GR in the absence of steroid may have masked induction of the reporter gene by low levels of steroid. Therefore although GR has nM affinity for B, activation of the reporter gene in the presence of 1nM B is not apparent. The mechanism of MMTV-LTR luciferase activation by GR in the absence of steroid is not known. However it is likely to be a phenomenon peculiar to the transfection method. For instance, it has been reported that the magnitude of several transcritional responses elicited by transfected GR is approximately proportional to the number of GR molecules per cell (Vanderbilt et al., 1987). Thus if there are few GR molecules per cell, then MMTV-LTR activation by GR could be low. Alternatively, it has been shown that nuclear factor I (NFI) is required for efficient transcription of the MMTV-LTR promoter in JEG3 cells even after GR transfection and treatment with glucocorticoids (Bruggemeir et al., 1990). JEG3 cells contain low levels of NFI, and transcription of the MMTV-LTR promoter is enhanced by co-transfection with NFI cDNA (Bruggemeir et al., 1990). Thus levels of NF1 or possibly other transcription factors in COS-7 cells may affect the transcription of MMTV-LTR luciferase. Interestingly, phenol red has been shown to have oestrogenic properties, and at the levels found in normal growth mediums, this is sufficient to bind to oestrogen receptors (Berthois et al., 1986). Whether phenol red in normal growth medium has any effects on GR is not known.

In a recent study looking at the regulation of 11ß-OHSD in intact human foreskin fibroblasts in culture (Hammami & Siiteri, 1991), both dehydrogenase and reductase activities of 11ß-OHSD were demonstrated, the dehydrogenase component being 5-10 times lower than its reductase counterpart. However when [$^3$H] cortisol was used as substrate, in the presence of excess cortisone, higher dehydrogenase activity was observed suggesting that in the absence of cortisone, dehydrogenase activity was

underestimated due to the rapid back-conversion of cortisone to cortisol. It is possible that the reductase activity measured in COS-7 and CV-1 cells could also be reversible and the measured activity was the result of a dynamic equilibrium between dehydrogenase and reductase activities but this was not investigated further since the aim of this study was to determine the direction of 11ß-OHSD activity in intact mammalian cells.

It is probable that the overall direction of the reversible hepatic 11ß-OHSD enzyme reaction is reduction (ie activation of inert glucocorticoids) (Bush et al., 1969). In addition, results presented in this chapter provide further evidence to suggest that the 'liver-type' 11ß-OHSD acts predominantly in the reductase direction in at least some intact mammalian cells. However in intact liver, the direction in specific subregions is not known, and there are several functions of 11ß-OHSD which could not be rationally explained if the 'liver-type' 11ß-OHSD was purely reductase. For example, in rats 11ß-OHSD antigen is expressed in leydig cells from puberty to adult when testosterone secretion is apparent (Philips et al., 1989). This 11ß-OHSD species was shown to be identical to the 'liver-type' isoform by Western blotting (Philips et al., 1989). However, corticosteroids directly suppress testosterone production in rats, leading to the suggestion that 11ß-OHSD inactivates glucocorticoids and allows testosterone synthesis to take place. Therefore if 11ß-OHSD activated inert glucocorticoids, testosterone production in rats would be attenuated and sexual development would be inhibited. Similarly in Chapter 3, it was shown that expression of hippocampal 11ß-OHSD activity and mRNA are increased by glucocorticoids. Hippocampal neurones are very sensitive to chronic excess or depletion of glucocorticoids, and we have suggested that 11ß-OHSD regulates glucocorticoid access to receptors in the hippocampus. However, if hippocampal 11ß-OHSD which is also the same isoform as the 'liver-type' enzyme, was purely a reductase in intact cells, then hippocampal neurones could be exposed to excess glucocorticoids possibly leading to brain dysfunction and neuronal cell death. Glucocorticoids are also required for lactation. 11ß-OHSD in the mammary gland decreases as pregnancy progresses and therefore has been proposed to inactivate glucocorticoids and prevent premature milk production in females (Quirk et al., 1990a; 1990b). As a reductase, 11ß-OHSD could not fulfil this proposed role. It is therefore likely that the overall direction of 11ß-OHSD activity is determined at a tissue-specific level, with reductase activity predominating in some cell types (eg COS-7, liver) and dehydrogenase activity predominating in others (eg leydig cells, hippocampus and the mammary gland). In addition it is possible that dehydrogenase and reductase activities may both be expressed in some cell types as demonstrated in CHO cells (Agarwal et al., 1989).

At the cellular level several mechanisms which could regulate the overall direction of 11ß-OHSD activity. The most likely is the glycosylation state of 11ß-OHSD; the 'liver-type' 11ß-OHSD has two putative N-linked glycosylation sites, and inhibition of glycosylation selectively inhibits dehydrogenase activity leaving reductase unaffected (Agarwal et al., 1989). This mechanism could act in a cell-specific or even compartment-specific level to give reductase activity in some cells or both in others. However it unlikely to be the direction determining mechanism in intact COS-7 cells. Although 11ß-OHSD activity is predominantly reductase in intact COS-7 cells, homogenates of the same cells readily express 11ß-dehydrogenase activity indicating that the cells are capable of glycosylating 11ß-OHSD, and yet intact cells predominantly activate glucocorticoids. Secondly, the concentration of NADP and NADPH may determine the relative proportion of dehydrogenase and reductase activity, with NADP favouring dehydrogenase activity and NADPH favouring the reductase direction (Agarwal et al., 1989). In single cells metabolism may favour a high NADP/NADPH ratio while other cells favour a low NADP/NADPH ratio perhaps leading to rapid local changes in the direction of 11ß-OHSD activity. However so many cellular reactions occur in which enzymes require NADP and NADPH as co-factors, that it is unlikely the pyridine nucleotide concentrations will change by sufficient amounts to be the sole regulator of a single enzyme for any length of time. Thirdly, in theory pH (Deckx & De Moore, 1966) may also have a role in determining the direction of 11ß-OHSD activity. However although pH may change sufficiently within a particular cellular compartment, pH is unlikely to change within cells by sufficient amounts to affect the 11-hydroxy/11-oxo ratio to any significant degree (Lakshmi & Monder, 1985; Monder & Shackleton, 1984). It is also possible that the glucocorticoid levels in tissues at particular times could regulate the direction of 11ß-OHSD activity such that when active glucocorticoid levels are low, the enzyme is predominantly reductase, and vice versa. In support of this hypothesis, it has recently been shown that dexamethasone treatment of a rat hepatoma cell line containing MMTV-glycoproteins led to increased expression of the MMTV-glycoprotein gene due to increased stability of the gene by a post-translational modification mechanism (Goodman & Firestone, 1993). Whether glucocorticoids also increase the stability of 11ß-OHSD and in particular dehydrogenase verus reductase components of 11ß-OHSD, remains to be determined.

An interesting question arising from this study is the possible physiological relevance of 11ß-reductase activity particularly in kidney derived cell lines such as COS-7. In kidney, 11ß-OHSD 2 is believed to rapidly inactivate all glucocorticoids passing through the distal nephron. However, 'liver-type' 11ß-OHSD mRNA (Yau et

al., 1991) and immunoreactivity (Edwards et al., 1988) is also found in kidney tubules. In addition it is known that the renal tubule contains glucocorticoid receptors (Farman et al., 1991; Katz, 1990) which mediate glucocorticoid-specific effects in the kidney including effects on renal haemodynamics, acid and water excretion, gluconeogenesis, and sodium-potassium ATPase (Katz, 1990; Kinsella, 1990). Therefore, complete inactivation of glucocorticoids may be detrimental to normal kidney function, and may explain the relevance of 11ß-reductase activity even in kidney-derived cell lines.

In conclusion, the rat 'liver-type' 11ß-OHSD cDNA transiently transfected into COS-7 mammalian cells acts predominantly as a reductase as shown by conversion of [$^3$H] 11-dehydrocorticosterone to [$^3$H] corticosterone in intact cells and activation of a glucocorticoid-responsive gene by the inactive 11-dehydrocorticosterone only in the presence of 11ß-OHSD. In addition it has been shown that 'liver-type' 11ß-OHSD can alter access of glucocorticoids to glucocorticoid receptors in an intact cell system.

# CHAPTER 7

# DISCUSSION

---

11ß-OHSD was first shown to be a potential mechanism for regulating glucocorticoid access to renal mineralocorticoid receptors in 1988, when it was shown that inhibition of 11ß-OHSD abolished selective aldosterone binding to renal mineralocorticoid receptors (Edwards et al., 1988; Funder et al., 1988). Since that time our understanding of the physiology of 11ß-OHSD has developed rapidly to provide solutions to many questions, and of course to reveal many more problems and possible research proposals for the future.

It is now known that 11ß-OHSD is found in numerous tissues in close proximity to both mineralocorticoid and glucocorticoid receptors strongly suggesting that 11ß-OHSD may influence corticosteroid-dependent processes in all tissues in which it is found. Thus in mineralocorticoid responsive tissues such as kidney, 11ß-OHSD is proposed to inactivate glucocorticoids therefore allowing selective access of aldosterone to the otherwise non-selective mineralocorticoid receptors. In contrast, in glucocorticoid receptor rich tissues, or tissues expressing both types of corticosteroid receptor, 11ß-OHSD is thought to play a more regulatory role to optimise glucocorticoid levels by activating or inactivating circulating glucocorticoid levels appropriately.

The obvious question which arises from these mechanisms of action is how 11ß-OHSD could be regulated to play such tissue-diverse roles. Part of the answer to this question has been revealed from several studies recently indicating the existence of a second isoform of 11ß-OHSD which is NAD-dependent and found in kidney (Rusvai et al., 1993) and a similar or the same NAD-dependent isoform found in human placenta (Brown et al., 1993). Characterisation and partial purification of human placental 11ß-OHSD has revealed that it encodes purely dehydrogenase activity in the absence of reductase, and has nM affinity for cortisol and corticosterone. Thus this isoform of 11ß-OHSD is highly suited to the protective role required in both placenta and kidney. Definitive characterisation of human placental and/or renal 11ß-OHSD will however only be possible with purification of the protein(s) and cDNA(s) and gene(s) which encode them.

In contrast to mineralocorticoid responsive tissues, tissues expressing both corticosteroid receptors, or predominantly glucocorticoid receptors, generally express 'liver-type' 11ß-OHSD activity, mRNA expression and immunoreactivity which in contrast to the NAD-dependent isoform is thought to be a reversible enzyme. Indeed previous studies have shown that 'liver-type' 11ß-OHSD cDNA encodes both reductase, and dehydrogenase activities (Agarwal et al., 1989; 1990). The same studies also suggested that glycosylation status and co-factor concentrations regulated the predominant direction of 11ß-OHSD activity such that glycosylation and high NADP concentrations favour dehydrogenase activity, while the non-glycosylated, high NADPH state favours reductase activity (Agarwal et al., 1990). However transfection studies presented here would tend to disagree with the importance of the glycosylation state as a direction contolling mechanism in intact cells, since 11ß-OHSD activity in homogenates can be driven in the dehydrogenase direction although reductase activity predominates in the intact cells. Nevertheless it would be interesting to look the influence of glycosylation status on the activity of 11ß-OHSD by site-directed mutagenesis of one or other or both putative N-linked glycosylation sites on the 'liver-type' 11ß-OHSD cDNA followed by transfection into cells and measurement of 11ß-OHSD activity in both the dehydrogenase and reductase directions. It should also be possible to determine the influence of co-factor status on the reversibility of 11ß-OHSD by manipulating NADP/NADPH levels or measuring the relative dehydrogenase and reductase activities of 11ß-OHSD in primary cultures of cells such as hepatocytes which are known to have high co-factor reserves, and hippocampal neurones which have much lower co-factor levels. An added complication in these experiments which was also noted in the transfection studies here, would be the effect of different cell types on the reversibility of 11ß-OHSD. Thus although COS-7 cells expressed no 11ß-dehydrogenase activity even when transfected with 10µg 11ß-OHSD which produced high levels of 11ß-reductase activity, CV-1 cells (from which COS-7 cells are derived) did convert a little corticosterone to 11-dehydrocorticosterone and this effect was optimal in cells transfected with 3µg 11ß-OHSD plasmid. Thus the predominant direction of 11ß-OHSD may also be regulated in a cell-specific manner, and it would be enlightening to look at various other cell lines transfected with 11ß-OHSD plasmids to determine if this was indeed the case. Finally, glucocorticoid levels themselves could possibly regulate the direction of 11ß-OHSD activity. In Chapter 3, I demonstrated that 11ß-OHSD activity and 'liver-type' mRNA expression were up-regulated in liver and hippocampus by both dexamethasone and chronic stress. However how these manipulations affect the reductase component of 11ß-OHSD, or the direction of 11ß-OHSD activity *in vivo* is not known. Transfection experiments were carried out at

only one glucocorticoid concentration, and it may therefore be interesting to look at the influence of different levels of glucocorticoids on the direction of 11ß-OHSD activity in intact cells.

Studies on the regulation of 11ß-OHSD in this thesis have led to several interesting findings. Firstly 11ß-OHSD is regulated in a tissue-specific manner. Thus although liver and hippocampus are regulated by glucocorticoids, 11ß-OHSD in kidney was unaffected by adrenal steroid manipulations. In contrast, hippocampal 11ß-OHSD was unaffected by gonadal steroids or growth hormone, whereas 11ß-OHSD in liver and kidney showed potent regulation by both. It is therefore likely that tissue-specific factors affect the actions of 11ß-OHSD *in vivo*. One of the most compelling pieces of evidence for this is the demonstration that 11ß-OHSD in liver and hippocampus predominantly utilise the same transcription start-site, yet 11ß-OHSD in liver is repressed by oestrogen, while hippocampal 11ß-OHSD is unaffected by oestrogen manipulations. In addition, oestrogen has free access to the brain, oestrogen receptors have been demonstrated in hippocampus, and it has been shown here that oestrogen may have a direct effect on 11ß-OHSD in liver. Tissue-specific factors in liver and hippocampus which bind to the 'liver-type' 11ß-OHSD could be measured in band shift assays, and the areas of DNA to which these putative factors bind could be determined by footprinting analyses.

11ß-OHSD activity in liver of control animals was high, and similar to levels observed following chronic dexamethasone treatment, while in hippocampus, 11ß-OHSD activity in sham-operated controls was low, and similar to levels expressed in adrenalectomised animals. This suggests a fundamental difference in the manner in which liver and hippocampus are exposed to glucocorticoids under basal conditions. This may be a function of the reversiblity of 'liver-type' 11ß-OHSD which is thought to be a reductase in liver (Bush et al., 1968), and is proposed to be dehydrogenase in hippocampus (Moisan et al., 1990a). However the predominant direction of 11ß-OHSD *in vivo* is not known, and measuring this is technically difficult. However initial liver perfusion studies have indicated that 11ß-OHSD activity in liver is indeed predominantly reductase (Pendek et al., unpublished observations). Manipulation of brain slices through the hippocampus in a similar way, by application of either [$^3$H]-corticosterone or [$^3$H] 11-dehydrocorticosterone to one side of the brain slice and collection of the tritiated products on the other tissue face may produce slightly more physiological data about the direction of 11ß-OHSD activity in hippocampus *in vivo*.

It has been demonstrated here, and previously, that hepatic 11ß-OHSD is expressed in a sexually dimorphic manner with higher levels of activity present in male than female rats. What is the physiological significance of lower 11ß-OHSD in

female liver? Since 11ß-OHSD in liver is thought to be predominantly reductase, lower 11ß-OHSD activity in females may explain the higher incidence of glucocorticoid-responsive inflammatory disorders of the liver in females. In addition, sexual dimorphism of other steroid metabolising and drug metabolising enzymes may explain the differences in drug and alcohol metabolism between males and females, but why this sexual dimorphism exists is not known. However, a possible way of determining the importance of 11ß-OHSD in normal liver function was revealed from sex steroid manipulation studies. Although the silastic capsules containing oestradiol had been reported to produce oestrogen levels not significantly different from those in control female rats, oestradiol replacement significantly repressed 11ß-OHSD in liver below levels found in control female rats indicating that this dose of oestradiol is in fact pharmacological and not physiological. Thus 10d oestradiol treatment almost completely abolished 'liver-type' 11ß-OHSD mRNA expression, and significantly repressed 11ß-OHSD activity. It is assumed that longer treatment periods would eventually abolish both 11ß-OHSD activity and mRNA expression. At this point it would be very interesting to look at how liver function is affected by the absence of 11ß-OHSD by looking at the expression of glucocorticoid-responsive genes such as angiotensinogen, tyrosine amino transferase (TAT) and phosphoenol pyruvate carboxykinase (PEPCK).

The sexual dimorphism of hepatic 11ß-OHSD activity and mRNA expression was revealed to be due at least in part by the female-specific pattern of GH secretion. As expected, GH did not affect 11ß-OHSD in hippocampus probably partly due to problems of access from the periphery to the brain. However in light of the differential regulation of hippocampus and liver by oestrogen, it may be worthwhile to look at possible regulation of 11ß-OHSD by GH to further characterise tissue-specific regulation of 11ß-OHSD. This could be achieved by intracerebroventricular cannulation followed by the same dose of GH administered in either continuous or pulsatile patterns, followed by measurement of 11ß-OHSD activity and mRNA expression.

*In vivo* manipulation of steroids and hormones do not always provide definitive information on the mechanism of action. Thus although female-pattern GH represses hepatic 11ß-OHSD, whether this is due to a direct action of GH or requires the presence of other factors such as insulin-like growth factors is not known. However this information could be obtained by looking at primary hepatocytes in culture treated with GH alone or in combination with other factors followed by measurement of changes in 11ß-OHSD mRNA levels.

Finally as a direct result of regulation studies in this thesis, it would be informative to know how the isoform of 11ß-OHSD found in kidney which is

potently induced by oestrogen, but appears to be unaffected by GH, is regulated, both in kidney and in other tissues which contain the same isoform. However apart form looking at levels of NAD-dependent activity following the same hormonal manipulations, this is not possible at the moment and will require complete purification and cloning of the corresponding cDNA before definitive studies can be performed.

In summary, these studies have demonstrated tissue-specific regulation of 11ß-OHSD in rat liver, kidney and hippocampus. It has been shown that liver and hippocamus are probably exposed to different glucocorticoid levels under basal conditions, and this may be a function of the predominant direction of 11ß-OHSD activity in the two tissues. In addition, hepatic 11ß-OHSD demonstrates sexually dimorphic expression due at least in part to the sexually differentiated patterns of GH secretion in male and female rats. Sex steroid and growth hormone manipulation studies also provided further evidence suporting the presence of a second isoform of 11ß-OHSD found in kidney. Finally, it was shown that 'liver-type' 11ß-OHSD cDNA encodes reductase but not dehydrogenase activity in at least some intact mammalian cells, and as such may regulate the access of active glucocorticoids to glucocorticoid receptors in some cell types and tissues.

## REFERENCES


Abe H, Molitch ME, van Wyk J, Underwood LE. (1983) Human growth hormone and somatomedian C supress the spontaneous release of growth hormone in unanesthatised rats. *Endocrinol* **113**: 1319-1324.

Abramovitz M, Carriero R, Murphy BEP. (1984) Investigation of factors influencing 11ß-hydroxysteroid dehydrogenase (EC 1.1.1.146) activity in midgestation human fetal lung monolayer and explant cultures. *J Steroid Biochem* **21**: 677-683.

Abramovitz M, Branchaud CL, Murphy BEP. (1982) Cortisol-cortisone conversion in human foetal lung: Contrasting results using explant and monolayer cultures suggest that 11ß-hydroxysteroid dehydrogenase (EC 1.1.1.146) comprises two enzymes. *J Clin Endocrinol Metab* **54**: 563-568.

Agarwal AK, Tusie-Luna M-T, Monder C, White PC. (1990) Expression of 11ß-hydroxysteroid dehydrogenase using recombinant vaccinia virus. *Mol Endocrinol* **4**: 1827-1832.

Agarwal AK, Monder C, Eckstein B, White PC. (1989) Cloning and expression of rat cDNA encoding corticosteroid 11dehydrogenase. *J Biol Chem* **264**: 18939-18943.

Albert DJ, Jonik RH, Walsh ML. (1992) Interaction of estradiol, testosterone and progesterone in the modulation of hormone-dependent aggression in the female rat. *Physiol Behav* **52**: 773-779.

Alpert LC, Brawer Jr, Patel YC, Reichlen S. (1976) Somatostatinergic neurons in anterior hypothalamus: immunohistochemical localisation. *Endocrinol* **98**: 255-262.

Andersson S, Berman DM, Jenkins EP, Russell DW. (1991) Deletion of steroid 5α-reductase 2 gene in male pseudohermaphroditism. *Nature* **354**: 159-161.

Antoni FA. (1993) Vasopressinergic control of pituitary adrenocorticotropin secretion comes of age. *Frontiers Neuroendocrinol* **14**: 76-122.

Antoni FA. (1986) Hypothalamic control of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor. *Endocrine Rev* **7**: 351-378.

Archer TK, Cordingley MG, Wolford RG, Hager GI. (1991) Transcription factor access is mediated by accurately positioned nucleosomes on the mouse mammary tumour virus promoter. *Mol Cell Biol* **11**: 688-698.

Argente J, Chowen-Breed JA, Steiner RA, Clifton DK. (1990) Somatostatin messenger RNA in hypothalamic neurons is increased by testosterone through activation of androgen receptors and not by aromatization to estradiol. *Neuroendocrinol* **52**: 342-349.

Argentin S, Nemer M, Drouin J, Scott GK, Kennedy BP, Davies PL. (1985) The gene for rat atrial natriuretic factor. *J Biol Chem* **260**: 4568-4571.

Armanini D, Karbowiak I, Funder JW. (1983) Affinity of liquorice derivatives for mineralocorticoid and glucocorticoid receptors. *Clin Endocrinol* **19**: 609-612.

Arriza JL, Simerly RB, Swanson LW, Evans RM. (1988). The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. *Neuron* **1**: 887-900.

Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, Evans RM. (1987). Cloning of the human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science* **237**: 268-275

Baggia S, Albrecht ED, Pepe GJ. (1990) Regulation of 11ß-hydroxysteroid dehydrogenase activity in baboon placenta by estrogen. *Endocrinol* **126**: 2742-2748.

Baird CW, Bush IE. (1960) Cortisol and cortisone content of amniotic fluid from diabetic and non diabetic women. *Acta Endocrinol* **34**: 97-104.

Baker ME. (1990a) Sequence similarity between Pseudomonas dehydrogenase, part of the gene cluster that metabolises polychlorinated biphenyls and dehydrogenases involved in metabolism of ribitol and glucitol and synthesis of antibiotics and 17ß-oestradiol, testosterone and corticosterone. *Biochem J* **267**: 839-841.

Baker ME. (1990b) A common ancestor for human placental 17ß-hydroxysteroid dehydrogenase, *Streptomyces coelicolor* act III protein, and *Drosophila melongaster* alcohol dehydrogenase. *FASEB J* **4**: 222-226.

Ballard PL. (1987) Glucocorticoid regulation of lung maturation. *Mead Johnson Symp Perinat Dev Med* 22-27.

Balmain A, Krumlauf R, Vass JK, Birnie GD. (1982) Cloning and characterisation of the abundant cytoplasmic 7S RNA from mouse cells. *Nucl Acids Res* **10**: 4259-4277.

Bambino TH, Hseuh AJW. (1981) Direct inhibitory effects of glucocorticoids upon testicular leutinizing hormone receptor and steroidogenesis in vivo and in vitro. *Endocrinol* **108**: 2142-2148.

Barker DJP, Bull AR, Osmond C, Simmonds SJ. (1990) Fetal and placental size and risk of hypertension in adult life. *Br Med J* **301**: 259-263.

Barker DJP, Osmond C, Winter PD, Margetts B, Simmonds SJ. (1989a) Weight in infancy and death from ischaemic heart disease. *Lancet* **ii**: 577-580.

Barker DJP, Osmond C, Golding J, Kuh D, Wadsworth MEJ. (1989b) Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *Br Med J* **298**: 564-567.

Bastl CP. (1988) Effect of spironolactone on glucocorticoid-induced colonic cation transport. *Am J Physiol* **255**: F1235-F1242.

Bastl CP. (1987) Regulation of cation transport by low doses of glucocorticoids in vivo adrenalectomised rat colon. *J Clin Invest* **80**: 348-356.

Beato M. (1989) Gene regulation by steroid hormones. *Cell* **56**: 335-344.

Beitens IZ, Bayard F, Ances IG, et al. (1973) The metabolic clearance rate, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy near term. *Pediatr Res* **7**: 509-519.

Benediktsson R, Lindsay R, Noble J, Seckl JR, Edwards CRW. (1993) Glucocortioid exposure in utero: a new model for adult hypertension. *Lancet* **341**: 339-341.

Benediktsson R, Yau JLW, Low SC, Brett L, Cooke BE, Edwards CRW, Seckl JR. (1992) 11ß-hydroxysteroid dehydrogenase in the rat ovary: high expression in the oocyte. *J Endocrinol* **135**: 53-58.

Berg JM. (1989) DNA binding specificity of steroid receptors. *Cell* **57**: 1065-1068.

Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. (1986) Phenol red in tissue culture media is a weak estrogen: Implications concerning the study of estrogen-responsive cells in culture. *Proc Natl Acad Sci USA* **83**: 2496-2500.

Bettini E, Pollio G, Santagati S, Maggi A. (1992) Estrogen receptor in rat brain: Presence in the hippocampal formation. *Neuroendocrinol* **56**: 502-508.

Birge CA, Peake GT, Mariz IK, Daughaday WH. (1967) Radio-immunoassayable growth hormone in the rat pituitary gland; effects of age, sex and homonal status. *Endocrinol* **81**: 195.

Boggaram V, Mendelson CR. (1988) Transcriptional regulation of the gene encoding the major surfactant protein (SP-A) in rabbit fetal lung. *J Biol Chem* **263**: 19060-19065.

Bonvalet J-P, Doignon J, Biot-Chaband M, Pradells P, Farman N. (1990) Distribution of 11ß-hydroxysteroid dehydrogenase along the rabbit nephron. *J Clin Invest* **86**: 832-837.

Borst JGG, Ten holt SP, De Vries LA. (1953) Synergistic action of liquorice and cortisone in Addison's and Simmond's disease. *Lancet* **i**:657-663.

Brazeau P, Vale W, Burgus R, Ling N, Butcher M, Rivier J, Guillemin R. (1973) Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science* **179**: 77-79.

Brem AS, Matheson KL, Conca T, Morris DJ. (1989) Effect of carbenoxolone on glucocorticoid metabolism and Na transport in toad bladder. *Am J Physiol* **257**: F700-F704.

Brinton RE, McEwen BS. (1987). Regional distinctions in the regulation of type I and type II adrenal steroid receptors in the central nervous system. *Neurosci Res Comm* **2**: 37-45.

Brown RW, Chapman KE, Edwards CRW, Seckl JR. (1993a) Human placental 11ß-hydroxysteroid dehydrogenase: Evidence for and partial purification of a distinct NAD-dependent isoform. *Endocrinol* **132**: 2614-2621.

Brown R.W., Chapman K.E., Edwards C.R.W., Seckl J.R. (1993b). A novel high affinity 11ß-hydroxysteroid dehydrogenase in placenta and kidney? *J Endocrinol* **137**: (suppl) P18.

Bugge TH, Pohl J, Lonnoy O, Stunnenberg HG. (1992) RXRα, a promiscuous partner of retinoic acid and thyroid hormone receptors. *EMBO J* **11**: 1409-1418.

Bruggemeier U, Rogge L, Winnacker E-L, Beato M. (1990) Nuclear factor I acts as a transcription factor on the MMTV promoter but competes with steroid hormone receptors for DNA binding. *EMBO J* **9**: 2233-2239.

Bullock P, Gemzik B, Johnson D, Thomas P, Parkinson A. (1991) Evidence from dwarf rats that growth hormone may not regulate the sexual differentiation of liver cytochrome P450 enzymes and steroid 5α-reductase. *Proc Natl Acad Sci USA* **88**: 6868-6872.

Bush IE. (1969) 11ß-hydroxysteroid dehydrogenase. Contrast between studies in vivo and studies in vitro. *Adv Biosci* **3**: 23-40.

Bush IE, Hunter SA, Meigs RA. (1968) Metabolism of oxygenated steroids. Metabolism in vitro by preparation of liver. *Biochem J* **107**: 239-258.

Canavan JP, Goldspink DF. (1988) Maternal diabetes in rats II: effects on fetal growth and protein turnover. *Diabetes* **37**: 1671-1623.

Carlson HE, Mariz IK, Daughaday WH. (1974) Thyrotropin-releasing hormone stimulation and somatostatin inhibition of growth hormone secretion from perfused rat adenohypophyses. *Endocrinol* **94**: 1709-1713.

Carlstedt-Duke J, Okret S, Wrange O, Gustafsson J-A. (1982) Immunochemical analysis of the glucocorticoid receptor: identification of a third domain separate from the steroid-binding and DNA-binding domains. *Proc Natl Acad Sci USA* **79**: 4260-4264.

Castello R, Schwarting R, Muller C, Heirholzer K. (1989) Immunohistochemical localisation of 11ß-hydroxysteroid dehydrogenase in rat kidney with monoclonal antibody. *Renal Physiol Biochem* **12**: 320-327.

Charlton HM, Clark RG, Robinson ICAF, Porter Goff AE, Cox BS, Bugnon C, Bloch BA. (1988) Growth hormone deficient dwarfism in the rat: a new mutation. *J Endocrinol* **119**: 51-58.

Cheng K-C, Schenkman JB. (1982) Purification and characterisation of two constitutive forms of rat liver microsomal cytochrome P-450. *J Biol Chem* **257**: 2378-2385.

Chomczynski P, Sacchi N. (1987) Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**: 156-159.

Chua SC, Szabo P, Vitek A, Grzeschik K-H, John M, White PC. (1987) Cloning of cDNA encoding steroid 11ß-hydroxylase P450c11. *Proc Natl Acad Sci USA* **84**: 7193-7197.

Clark RG, Carlsson LMS, Robinson ICAF. (1987) Growth hormone secretory profiles in conscious female rats. *J Endocrinol* **114**: 399-407.

Chomczynski P, Sacchi N. (1987) Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**: 156-159.

Chua SC, Szabo P, Vitek A, Grzeschik K-H, John M, White PC. (1987) Cloning of cDNA encoding steroid 11ß-hydroxylase P450c11. *Proc Natl Acad Sci USA* **84**: 7193-7197.

Clark RG, Carlsson LMS, Robinson ICAF. (1987) Growth hormone secretory profiles in conscious female rats. *J Endocrinol* **114**: 399-407.

Clark RG, Robinson ICAF. (1985) Growth hormone responses to multiple injections of a fragment of human growth hormone-releasing factor in conscious rats. *J Endocrinol* **106**: 281-289.

Clark RG, Jansson JO, Isaksson O, Robinson ICAF. (1985) Intravenous growth hormone: Growth responses to patterned infusions. *Endocrinol* **104**: 53-61.

Cohen SI. (1980) Cushing's syndrome: A psychiatric study of 29 patients. *Br J Psych* **136**: 120-124.

Coiro V, Braverman LE, Christianson D, Fang SL, Goodman HM. (1979) Effect of hypothyroidism and thyroxine replacement on growth hormone in the rat. *Endocrinol* **105**: 641-646.

Cooney AJ, Tsai SY, O'Malley BW, Tsai M-J. (1992) Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTCA response elements allowing COUP-TF to repress hormonal induction of the vitamin D3, thyroid hormone and retinoic acid receptors. *Mol Cell Biol* **12**: 4153-4163.

Cunnah D, Jessop DS, Besser GM, Rees L. (1987) Measurement of circulating corticotropin-releasing factor in man. *J Endocrinol* **113**: 123-131.

Cupps TR, Gerrard TL, Falkoff JM, Waalen G, Fauci AS (1985) Effects of in vitro corticosteroids on B cell activation, proliferation, and differentiation. *J Clin Invest* **75**: 754-761.

Danielson M, Northrop JP, Jonklass J, Ringold GM. (1987) Domains of the glucocorticoid receptor involved in specific and non-specific deoxyribonucleic acid binding, hormone activation, and transcriptional enhancement. *Mol Endocrinol* **11**: 816-822.

Danielson M. (1991) Structure and function of the glucocorticoid receptor. In "Nuclear Hormone Receptors" (ed Parker MG) pp 39-78. Academic Press, London, UK.

Danielson M, Hinck L, Ringold GM. (1989) Two amino acids within the knuckle of the first zinc finger specify DNA response element activation by the glucocorticoid receptor. *Cell* **57**: 1131-1138.

Deckx R, De Moor P. (1966) Study of the 11ß-hydroxysteroid dehydrogenase in vitro. I. Biochemical characterisation in spleen homogenates. *Pflugers Arch* **289**: 59.

De Franco DB, Qi M, Borror KC, Garabedian MJ, Brautigan DL. (1991) Protein phosphatase types I and/or 2A regulate nucleocytoplasmic shuttling of the glucocorticoid receptor. *Mol Endocrinol* **5**: 1215-1228.

De Gennaro V, Zoli M, Cocchi D, Maggi A, Marrama P, Agnati LF, Muller EE. (1989) Reduced growth hormone releasing factor (GHRF)-like immunoreactivity and GHRF gene expression in the hypothalamus of aged rats. *Peptides* **10**: 705-708.

De Kloet ER, Reul JMHM. (1987) Feedback action and tonic influences of corticosteroids on brain function: a concept arising from heterogeneity of brain receptor systems. *Psychoneuroendocrinol* **12**: 83-105.

De Wet JR, Wood KV, Deluca M, Helinsji DR, Subramani S. (1987) Firefly luciferase gene: Structure and expression in mammalian cells. *Mol Cell Biol* **7**: 725-37.

Donaldson LF, Seckl JR, McQueen DS. (1994) A discrete adjuvant-induced monoarthritis in the rat: effects of adjuvant dose. *J Neurosci Meths* (in press).

Dougherty TF, Berliner ML, Berliner DL. (1960) Hormonal influence on lymphocyte differentiation from RES cells. *Ann N Y Acad Sci* **88**: 78-82.

Drouin J, Sun YL, Chamberland M, Gauthier Y, De Lean A, Nemer M, Schmidt TJ. (1993) Novel glucocorticoid receptor complex with DNA element of the hormone repressed POMC gene. *EMBO J* **12**: 145-156.

Dunn JF, Nisula BC, Rodbard D. (1981) Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. *J Clin Endocrinol Metab* **53**: 58-68.

Dupperex H, Kenouch S, Gaeggeler HP, Seckl JR, Edwards CRW, Farman N, Rossier BC. (1993) Rat liver 11ß-hydroxysteroid dehydrogenase complementary deoxyribonucleic acid encodes oxoreductase activity in a mineralocorticoid-responsive toad bladder cell line. *Endocrinol* **132**: 612-619.

Durand B, Saunders M, Leroy P, Leid M, Chambon P. (1992) All-*trans* and 9-*cis* retinoic acid induction of CRABPII transcription is mediated by RAR-RXR heterodimers bound to DR1 and DR2 repeated motifs. *Cell* **71**: 73-85.

Eden S, Jansson J-O, Oscarsson J. (1987) Sexual dimorphism of growth hormone secretion. In "Growth Hormone: Basic and Clinical Aspects" (eds Isaksson O, Binder C, Hall K, Hokfelt B), pp 129-151, Excerpta Medica, Amsterdam.

Eden S, Eriksson E, Martin JB, Modigh K. (1981) Evidence for a growth hormone releasing factor mediating alpha-adrenergic influence on growth hormone secretion in the rat. *Neuroendocrinol* **33**: 24-27.

Eden S. (1979) Age- and sex-related differences in episodic growth hormone secretion in the rat. *Endocrinol* **105**: 555-560.

Edenberg HJ, Brown CJ. (1992) Regulation of human alcohol dehydrogenase genes. *Pharmacogenetics* **2**: 185-196.

Edwards CRW, Benediktsson R, Lindsay RS, Seckl JR. (1993) Dysfunction of placental glucocorticoid barrier: link between fetal environment and adult hypertension? *Lancet* **341**: 355-357.

Edwards CRW, Stewart PM, Burt D, Brett L, McIntyre MA, Sutanto WS, de Kloet ER, Monder C. (1988) Localisation of 11ß-hydroxysteroid dehydrogenase-tissue specific protector of the mineralocorticoid receptor. *Lancet* **ii**: 986-989.

Eikelboom R, Tannenbaum GS. (1983) Effects of obesity-inducing ventromedial hypothalamic lesions on pulsatile growth hormone and insulin secretion: evidence for the existence of a growth hormone-releasing factor. *Endocrinol* **112**: 212-219.

Eipper BA, Mains RE. (1980) Structure and biosynthesis of pro-adrenocorticotropin/endorphin and related peptides. *Endocrine Rev* **1**: 1-27.

Evans RM, Birnberg NC, Rosenfeld MG. (1982) Glucocorticoid and thyroid hormones transcriptionally regulate growth hormone gene expression. *Proc Natl Acad Sci USA* **79**: 7659-7663.

Evans RM, Arriza JL. (1989) A molecular mechanism for the actions of glucocorticoid hormones in the nervous system. *Neuron* **2**: 1105-1112.

Evans RM. (1988) The steroid and thyroid hormone receptor superfamily. *Science* **240**: 889-894.

Evans RM, Hollenberg SM. (1988) Zinc fingers: gilt by association. *Cell* **52**: 1-3.

Exton JH (1979) Regulation of gluconeogenesis by glucocorticoids. In "Glucocorticoid Hormone Action" (eds Baxter JD, Rousseau GG) pp535-546, Springer-Verlag, NY.

Fain JH. (1979) Inhibition of glucose transport in fat cells and activation of lipolysis by glucocorticoids. In "Glucocorticoid Hormone Action" (eds Baxter JD, Rousseau GG) pp547-560, Springer-Verlag, NY.

Farman N, Oblin ME, Lombes M, Delahaye F, Westphal HM, Bonvalet JP, Gasc JM. (1991) Immunolocalisation of gluco-and mineralocorticoid receptors in rabbit kidney. *Am J Physiol* **260**: C226-C233.

Fauci AS. (1979) Immunosuppressive and antiinflammatory effects of glucocorticoids. In "Glucocorticoid Hormone Action (eds Baxter JD, Rousseau GG) pp 449-465, Springer-Verlag, NY.

Facui AS, Dale DC. (1974) The effect of in vivo hydrocortisone on subpopulations of human lymphocytes. *J Clin Invest* **53**: 240-246.

Federoff HJ, Grabczyk E, Fishman MC. (1988) Dual regulation of GAP-43 gene expression by nerve growth factor and glucocorticoids. *J Biol Chem* **263**: 19290-19295.

Feinberg AP, Vogelstein B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**: 6.

Ferland L, Labrie F, Jobin M, Arimura A, Schally AV. (1976) Physiological role of somatostatin in the control of growth hormone and thyrotropin secretion. *Biochem Biophys Res Commun* **68**: 149-156.

Frankel AD, Pabo CO. (1988) Fingering too many proteins. *Cell* **53**: 675-670.

Frohman LA, Bernardis LL. (1968) Growth hormone and insulin levels in weanling rats with ventromedial hypothalamic lesions. *Endocrinol* **82**: 1125-1132.

Frohman LA, Bernardis LL, Kant KJ. (1968) Hypothalamic stimulation of growth hormone secretion. *Science* **162**: 580-581.

Fuller PJ, Verity K. (1990) Colonic sodium-potassium adenosine triphosphate subunit gene expression: ontogeny and regulation by adrenocortical steroids. *Endocrinol* **127**: 32-38.

Funder JW, Pearce PT, Smith R, Campbell J. (1989) Vascular type I aldosterone binding sites are physiological mineralocorticoid receptors. *Endocrinol* **125**: 2224-2226.

Funder JW, Pearce PT, Smith R, Smith AI. (1988) Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science* **242**: 583-585.

Funder JW. (1986) Adrenocorticoid receptors in the brain. In "Frontiers in Neuroendocrinology" (WF Ganong, L Martini, eds), vol 9, pp 169-189. Raven Press, New York.

Funder JW, Duval D, Meyer P. (1973) Cardiac glucocorticoid receptors: the binding of tritiated-dexamethasone. *Endocrinol* **93**: 1300-1308.

Gaeggeler H-P, Edwards CRW, Rossier BC. (1989) Steroid metabolism determines mineralocorticoid specificity in the toad bladder. *Am J Physiol* **257**: F690-F695.

Gaillard RC, Poffet D, Riondel AM, Saurat J-H. (1985) RU486 inhibits peripheral effects of glucocorticoids in humans. *J Clin Endocrinol Metab* **61**: 1009-1011.

Gallin JC, Jacobs LS, Fram DH, (1972) Acute effect of glucocorticoid on human sleep. *Nature* **237**: 398-399.

Garcia R, Debinski W, Gutkowska J, Kuchel O, Thibault G, Genest J, Canth M. (1985) Gluco- and mineralocorticoids may regulate the natriuretic effect and the synthesis and release of natriuretic factor by the rat atria in vivo. *Biochem Biophys Res Commun* **131**: 806-814.

Garrod O, Burnston RA. (1952) The diuretic response to ingested water in Addison's disease and panhypopituitarism and the effect of cortisone thereon. *Clin Soc Lond* **11**: 113-128.

Gaunt R, Gisoldi E, Herkner J, Howie NC. (1968) Sodium retention after adrenal enucleation: drug and salt appetite studies. *Endocrinol* **83**: 927-932.

Gaunt R, Renzie AA, Gisoldi E, Howie N, Renzi AA. (1967) A sodium retaining influence of enucleate rat adrenal glands. *Endocrinol* **81**: 1331-1337.

Ghosh D, Weeks CM, Grochulski P, Duax WL, Erman M, Rimsay RL, Orr JC. (1991) Three-dimensional structure of holo 3α, 20ß-hydroxysteroid dehydrogenase: A member of a short chain dehydrogenase family. *Proc Natl Acad Sci USA* **88**: 10064-10068.

Ghraf R, Vetter U, Zandveld JM, Schreifers H. (1975a) Organ specific ontogenesis of steroid metabolising activities in the rat I. 11ß and 17ß hydroxysteroid dehydrogenase. *Acta Endocrinol* (Copenhagen) **79**: 192-201.

Ghraf R, Lax ER, Hoff H-G, Schreifers H. (1975b) The role of the gonads and the hypophysis in the regulation of hydroxysteroid dehydrogenase activities in rat kidney. *Hoppe-Seylers Z Physiol Chem* **356**: 135-142.

Ghraf R, Hoff H-G, Lax ER, Schreifers H. (1975c) Organ specific ontogenesis of steroid metabolising activities in the rat I. 11ß and 17ß hydroxysteroid dehydrogenase. *Acta Endocrinol* (Copenhagen) **79**: 192-201.

Gibbons GH, Dzau VJ, Farhl ER, Barger AC. (1984) Interaction of signals influencing renin release. *Ann Rev Physiol* **46**: 291-308.

Giguere V, Hollenberg SM, Rosenfels MG, Evans RM. (1986) Functional domains of the human glucocorticoid receptor. *Cell* **46**, 645-652.

Gill GN. (1972) Progress in endocrinology and metabolism: mechanism of ACTH action. *Metabolism* **21**: 571-588.

Gillis S, Crabtree GR, Smith KA. (1979) Glucocorticoid-induced inhibition of T cell growth factor production. I. The effect on mitogen-induced lymphocyte proliferation. *J Immunol* **123**: 1624-1631.

Glock GE, McLean P. (1955) Levels of oxidised and reduced diphosphopyridine nucleotide and triphosphopyridine nucleotide in animal tissues. *Biochem J* **61**: 388-390.

Gluzman Y. (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23**: 175-182.

Gomez-Sanchez EP. (1991) What is the role of the central nervous system in mineralocorticoid hypertension? *Am J Hypertens* **4**: 374-381.

Goodfriend TL, Gibbons GH, Dzau VJ, Farhl ER, Barger AC. (1980) Interaction of signals influencing renin release. *Ann Rev Physiol* **46**: 291-308.

Goodman LJ, Firestone GL. (1993) Glucocorticoid-regulated stability of a constitutively expressed mouse mammary tumor virus glycoprotein. *Mol Endocrinol* **7**: 94-103.

Goodman RH, Rehfuss RP, Verhave M, Ventimiglia R, Low MJ. (1990) Somatostatin gene regulation - an overview. *Metabolism* **39**: (suppl 2): 2-5.

Gordon GG, Southren AL. (1977) Thyroid hormone effects on steroid hormone metabolism. *Bull N Y Acad Med* **53**: 241-259.

Gorman CM, Merlino GT, Willingham MC, Pastan I, Howard BH. (1982) The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells. *Proc Natl Acad Sci USA* **79**: 6777-6781.

Graham BS, Tucker WS Jr. (1984) Opportunistic infections in endogenous Cushing's syndrome. *Ann Intern Med* **101**: 334-338.

Green S, Cambon P. (1988) Nuclear receptors enhance our understanding of transcription regulation. *TIG* **4**: 309-313.

Greenberg BD, Bencen GH, Seilhamer JJ, Lewicki JA, Fiddes JC. (1984) Nucleotide sequence of the gene encoding human atrial natriuretic factor precursor. *Nature* **312**: 656-658.

Grosser BI. (1966) 11ß-hydroxysteroid metabolism by mouse brain and glioma 261. *J Neurochem* **13**: 475-478.

Guillemin R, Brazeau P, BohlenP, Esch F, Ling N, Wehrenberg WB. (1982) Growth hormone-releasing factor from a human pancreatic tumor that caused acromegaly. *Science* **218**: 585-587.

Guiochon-Mantel A, Lescop P, Christian-Maitre S, Loosfelt H, Perrot-Applanat M, Milgrom E. (1991) Nucleocytoplasmic shuttling of the progesterone receptor. *EMBO J* **10**: 3851-3858.

Gustafsson J-A, Eden S, Eneroth P, Hokfelt T, Isaksson D, Jansson J-O, Mode A, Norstedt G. (1983) Regulation of sexually-dimorphic hepatic steroid metabolism by the somatostatin-growth hormone axis. *J Steroid Biochem* **19**: 691-698.

Guzelian PS, Li D, Schuetz EG, Thomas P, Levin W, Mode A, Gustafsson J-A. (1988) Sex change in cytochrome P-450 phenotype by growth hormone treatment of adult rat hepatocytes maintained in a culture system on matrigel. *Proc Natl Acad Sci USA* **85**: 9783-9787.

Hahn TJ, Halstead LR, Teitelbaum SL, Hahn BH. (1979) Altered mineral metabolism in glucocorticoid-induced osteopenia. Effect of 25-hydroxyvitamin D administration. *J Clin Invest* **64**: 655-665.

Halvey J, Boulpaep EL, Budinger ME, Binder HJ, Hayslett JP. (1988) Glucocorticoids have a different action than aldosterone on target tissue. *Am J Physiol* **254**: F153-F158.

Hammami MM, Siiteri PK. (1991) Regulation of 11ß-hydroxysteroid dehydrogenase activity in human skin fibroblasts: enzymatic modulation of glucocorticoid action. *J Clin Endocrinol Metab* **73**: 326-334.

Handa M, Kondo K, Suzuki H, Saruta T. (1983) Urinary prostaglandin $E_2$ and kallikrein excretion in glucocorticoid hypertension in rats. *Clin Sci* **65**: 37-42.

Hard T, Kellenbach E, Boelens R, Maler BA, Dahlman K, Freedman LP, Carlstedt-Duke J, Yamamoto KR, Gustafsson J-A. (1990) Solution structure of the glucocorticoid receptor DNA-binding domain. *Science* **249**: 157-160.

Hausberger FX. (1958) Influence of insulin and cortisone on hepatic and adipose tissue metabolism of rats. *Endocrinol* **63**: 14-19.

Heller CL, Weisenberg LS, Orti E, de Nicola AF. (1983) Steps in glucocorticoid action in normal and diabetic rat placenta. *J Steroid Biochem* **31**: 119-123.

Hellman L, Nakada F, Zumoff B, Fukushima D, Bradlow HL, Gallagher TF. (1971) Renal capture and oxidation of cortisol in man. *J Clin Endocrinol Metab* **33**: 52-62.

Hellman L, Bradlow HL, Zumoff B, Gallagher TF. (1961) The influence of thyroid hormone administration on the 11ß-hydroxysteroid dehydrogenase mRNA expression in rat kidney. *J Clin Endocrinol Metab* **21**: 1231-1247.

Herbert DC, Sheridan PJ. (1983) Uptake and retention of sex steroids by the baboon pituitary gland - evidence of sexual dimorphism with respect to dihydrotestosterone. *Biol Reprod* **28**: 377-383.

Hertz P, Silberman M, Even L, Hochberg Z. (1989) Effects of sex steroids on the response of cultured rat pituitary cells to growth hormone-releasing hormone and somatostatin. *Endocrinol* **125**: 581-585.

Hoefenagels WHL, Kloppenberg PWC (1983) Antimineralocorticoid effects of dexamethasone in subjects treated with glycyrrhetinic acid. *J Hypertension* **1** (suppl 2): 313-315.

Hoffman DL, Baker BL. (1977) Effect of treatment with growth hormone or somatostatin in the median eminence of hypophysectomised rats. *Proc Soc Exp Biol Med* **156**: 265-271.

Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, Thompson EB, Rosenfeld MG, Evans RM. (1985) Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature* **318**: 635-641.

Hong SC, Levine L. (1976) Inhibition of arachadonic acid release from cells as a biochemical action of anti-inflammatory steroids. *Proc Natl Acad Sci USA* **73**: 1730-1734.

Hurlock B, Talalay P. (1959) Microsomal 3α and 11ß-hydroxysteroid dehydrogenases. *Arch Biochem Biophys* **80**: 469-470.

Jahn GA, Moguilewsky M, Houdebine L-M, Dijane J. (1987) Binding and action of glucocorticoids and mineralocorticoids in rabbit mammary gland. Exclusive participation of glucocorticoid type II receptors for stimulation of caesin synthesis. *Mol Cell Endocrinol* **52**: 205-212.

Jansson J-O, Frohman LA. (1987) Differential effects of neonatal and adult androgen exposure on the growth homone secretory pattern in male rats. *Endocrinol* **120**: 1551-1557.

Jansson J-O, Eden S, Isaksson O. (1985a) Sexual dimorphism in the control of growth hormone secretion. *Endocrine Rev* **6**: 128-150.

Jansson J-O, Ekberg S, Isaksson OPG, Mode A, Gustafsson J-A. (1985b) Imprinting of growth hormone secretion, body growth, and hepatic steroid metabolism by neonatal testosterone. *Endocrinol* **117**: 1881-1889.

Jansson J-O, Ekberg S, Isaksson OPG, Eden S. (1984a) Influence of gonadal steroids on age- and sex-related secretory patterns of growth hormone in the rat. *Endocrinol* **114**: 1287-1294.

Jansson J-O, Isaksson O, Mode A, Gustafsson J-A. (1984b) Influence of neonatal androgen secretion on plasma GH levels, body growth and hepatic steroid metabolism in adult male rats. 7th International Congress of Endocrinol:p88.

Jansson J-O, Carlsson L, Seeman H (1983) Estradiol - but not testosterone - stimulates the secretion of growth hormone in rats with the pituitary gland autotransplanted to the kidney capsule. *Acta Endocrinol* **103**: (suppl 256): 212.

Jansson J-O, Albertsson-Wikland K, Eden S, Thorngren K-G, Isaksson O. (1982a) Circumstantial evidence for a role of the secretory pattern of growth hormone in the control of body growth. *Acta Endocrinol* **99**: 24-30.

Jansson J-O, Albertsson-Wikland K, Eden S, Thorgren K-G, Isaksson O. (1982b) Effect of frequency of growth hormone administration on longitudinal bone growth and body weight in hypophysectomised rats. *Acta Physiol Scand* **114**: 261.

Jenson EV. (1990) Molecular mechanisms of steroid hormone action in the uterus. In "Uterine Function: Molecular and Cellular Aspects" (eds Carsten ME, Miller JD.) pp 315-359. Plenum Publishing Corp, NY.

John ME, John MC, Simpson ER, Waterman MR. (1985) Regulation of cytochrome P-450-11ß gene expression by adrenocorticotropin. *J Biol Chem* **260**: 5760-5767.

Jornvall H, Persson M, Jeffrey J. (1981) Alcohol and polyol dehydrogenases are both divided into two protein types, and structural properties cross-relate the different enzyme activities within each type. *Proc Natl Acad Sci USA* **78**: 4226-4230.

Kamatak T, Shimada M, Maeda K, Kato R. (1985) Pituitary regulation of sex-specific forms of cytochrome P-450 apoprotein levels in rat liver. *Biochem Biophys Res Commun* **130**: 1247-1253.

Katz AJ. (1990) Corticosteroid regulation of Na-K-ATPase along the mammalian nephron. *Semin Nephrol* **10**: 382-399.

Katz SH, Molitch M, McCann SM. (1969) Effect of hypothalamic implants of GH on anterior pituitary weight and GH concentration. *Endocrinol* **85**: 725-734.

King RJB. (1992) Effects of steroid hormones and related compounds on gene transcription. *Clin Endocrinol* **36**: 1-14.

Kinsella JL. (1990) Action of glucocorticoids on proximal tubule transport systems. *Semin Nephrol* **10**: 330-338.

Kliewer SA, Umesono K, Mangelsdorf DJ, Evans RM. (1992) Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. *Nature* **355**: 446-449.

Knopf JL, Gallagher JF, Held WA. (1983) Differntial multi-hormone regulation of the mouse major urinary protein gene family in the liver. *Mol Cell Biol* **3**: 2232-2240.

Koerner DR, Hellman L. (1964) Effect of thyroxine administration on the 11ß-hydroxysteroid dehydrogenases in rat liver and kidney. *Endocrinol* **75**: 592-601.

Koerner DR. (1966) 11ß-hydroxysteroid dehydrogenase of lung and testis. *Endocrinol* **79**: 935-938.

Komolov IS, Perez-Arce JA, Fedotov VP. (1980) The effects of estradiol on prolactin and growth hormone secretion in cultured pituitary cells from intact and ovariectomised rats. *Endokrinol* **75**: 278.

Kornel L, Kananarlapudi N, Travers T, Taff DJ, Patel N, Chen C, Baum RM, Raynor WJ. (1982) Studies of high affinity binding of mineralo- and glucocorticoids in rabbit aorta cytosol. *J Steroid Biochem* **16**: 245-264.

Kornel L. (1988) Disparate effects of glucocorticoids and mineralocorticoids on sodium and water transport in vascular smooth muscle. *Endocrine Soc 70th Ann Meet Prog Abstr*:99.

Kotas RV, Avery J. (1971) Accelerated appearance of pulmonary surfactant in the fetal rabbit. *J App Physiol* **30**: 358-361.

Kozak M. (1991) An analysis of vertebrate mRNA sequences:initiations of translational control. *J Cell Biol* **115**: 887-903.

Krakoff L, Nicolis G, Amsel B. Pathogenesis of hypertension in Cushing's syndrome. *Am J Med* **58**: 216-220.

Kramer RE, Gallant S, Brownie AC. (1980) Actions of angiotensin II on aldosterone biosynthesis in the rat adrenal cortex. *J Biol Chem* **255**: 3442-3447.

Kreiger DT. (1972) The central nervous system and Cushing's disease. *Mt Sinai J Med (NY)* **39**: 416-442.

Krook M, Marekov L, jornvall H (1990) Purification and structural characterisation of placental NAD+-linked 15-hydroxyporstaglandin dehydrogenase. The primary structure reveals the enzyme to belong to the short chain alcohol dehydrogenase family. *Biochem* **29**: 738-743.

Krozowski ZS, Obeyesekere V, Smith R, Mercer W. (1992) Tissue-specific expression of an 11ß-hydroxysteroid dehydrogenase with a truncated N-terminal domain - a potential mechanism for differential intracellular localization within mineralocorticoid target cells. *J Biol Chem* **267**: 2569-2574.

Krozowski Z, Stuchbery S, White PC, Monder C. Funder JW. (1990) Characterisation of 11ß-hydroxysteroid dehydrogenase gene expression: identification of multiple unique forms of messenger ribonucleic acid in the rat kidney. *Endocrinol* **129**: 17-21.

Krozowski ZS, Funder JW. (1983) Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have intrinsic steroid specificity. *Proc Natn Acad Sci USA* **80**: 6056-6060.

Kurlich L, Dhariwal APS, McCann SM. (1968) Stimulatory and inhibitory effects of purified hypothalamic extracts on growth hormone release from rat pituitary *in vitro*. *Endocrinol* **83**: 783-790.

Kurlich L, McCann SM. (1966) Influence of growth hormone (GH) on content of GH in the pituitaries of normal rats. *Proc Soc Exp Biol Med* **121**: 1114.

Lakshmi V, Sakai RR, McEwen BS, Monder C. (1991) Regional distribution of 11ß-hydroxysteroid dehydrogenase in rat brain. *Endocrinol* **128**: 1741-1748.

Lakshmi V, Monder C. (1988) Purification and characterisation of the corticosteroid 11ß-dehydrogenase component of the rat liver 11ß-hydroxysteroid dehydrogenase complex. *Endocrinol.* **123**: 2390-2398.

Lakshmi V, Monder C. (1985) Evidence for independent 11-oxidase and 11-reductase activities of 11ß-hydroxysteroid dehydrogenase: enzyme latency, phase transition and lipid requirements. *Endocrinol* **116**: 552-560.

Latif SA, Conca TJ, Morris DJ. (1990) The effects of the liquorice derivative, glycyrrhetinic acid, on hepatic 3α and 3ß-hydroxysteroid dehydrogenases and 5α and 5ß-reductase pathway of metabolism of aldosterone in male rats. *Steroids* **55**: 52-58.

Lax ER, Ghraf R, Shreifers, Voigt KH. (1979) The involvement of the thyroid and adrenal in the regulation of hepatic and renal steroid metabolism in the rat. *Hoppe-Seylers Z Physiol Chem* **360**: 137-143.

Lax ER, Ghraf R, Schriefers H. (1978) The hormonal regulation of hepatic microsomal 11ß-hydroxysteroid dehyrogenase activity in the rat. *Acta Endocrinol* **89**: 352-357.

Lefebvre P, Berard DS, Cordingley MG, Hager GL. (1991) Two regions of the mouse mammary tumour virus long terminal repeat regulate the activity of its promoter in mammary cell lines. *Mol Cell Biol* **11**: 2529-2537.

Legraverend C, Mode A, Wells T, Robinson ICAF, Gustafsson J-A. (1992) Hepatic steroid hydroxylating enzymes are controlled by the sexually dimorphic pattern of growth hormone secretion in normal and dwarf rats. *FASEB J* **6**: 711-718.

Leibovich SJ, Ross R. (1975) The role of the macrophage in wound repair: A study with hydrocortisone and antimacrophage serum. *Am J Pathol* **75**: 71-100.

Leid M, Kastner P, Lyons R, Nakshatri H, Saunders M, Zacharewski T, Chen YY, Staub A, Garnier J-M, Mader S, Chambon P. (1992) Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* **68**: 377-395.

Liley HG, Hawgood S, Wellenstein GA, Benson B, White RT, Ballard PL. (1987) Surfactant protein of molecular weight 28,000-36,000 in cultured human fetal lung; cellular localization and effect of dexamethasone. *Mol Endocrinol* **1**: 205-215.

Lundbland JR, Roberts JL. (1988) Regulation of the proopiomelanocortin gene expression in pituitary. *Endocrine Rev* **9**: 135-158.

Lopez Bernal A, Flint APF, Anderson ABM. (1980) 11ß-hydroxysteroid dehydrogenase activity (E.C.1.1.1.146) in human placenta and decidua. *J Steroid Biochem* **13**: 1081-1087.

Low SC, Assaad SN, Rajan V, Chapman KE, Edwards CRW, Seckl JR. (1993) Regulation of 11ß-hydroxysteroid dehydrogenase by sex steroids in vivo: further evidence for the existence of a second dehydrogenase in rat kidney. *J Endocrinol* **139**: 27-35.

Lugg MA, Nicholas TE. (1978) The effect of dexamethasone on the activity of 11ß-hydroxysteroid dehydrogenase in the foetal rabbit lung during the final stages of gestation. *J Pharm Pharmacol* **30**: 587-588.

Luisi BF, Xu, WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB. (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* **352**: 497-505.

MacGeoch C, Morgan ET, Cordell B, Gustafsson J-A. (1987) Growth hormone regulates expression of rat liver cytochrome P-450 15ß at a pretranscriptional level. *Biochem Biophys Res Commun* **143**: 782-788.

MacGeoch C, Morgan ET, Halpert J, Gustafsson J-A. (1984) Purification, characterisation and pituitary regulation of specific cytochrome P-450 15ß-hydroxylase from liver microsomes of untreated female rats. *J Biol Chem* **259**: 15433-15439.

MacPhee IAM, Antoni FA, Mason DW. (1989) Spontaneous recovery of rats from experimental encephalomyelitis is dependent on regulation of the immune system by endogenous adrenal corticosteroids. *J Exp Med* **169**: 431-445.

Mader S, Kumar V, de Verneuil H, Chambon P. (1989) Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. *Nature* **338**: 271-274.

Maiter D, Koenig JI, Kaplan LM. (1991a) Sexually dimorphic expression of the growth hormone-releasing hormone gene is not mediated by circulating gonadal hormones in the adult rat. *Endocrinol* **128**: 1709-1716.

Maiter D, Underwood LE, Martin JB, Koenig JI. (1991b) Neonatal treatment with monosodium glutamate: effects of prolonged growth hormone (GH)-releasing hormone deficiency on pulsatile GH secretion and growth in female rats. *Endocrinol* **128**: 1100-1106.

Marekov L, Krook M, Jornvall H. (1990) Prokaryotic 3α, 20ß-hydroxysteroid dehydrogenase is an enzyme for the 'short-chain, non-metalloenzyme' alcohol dehydrogenase type. *FEBS Letts* **266**: 51-54.

Marks R, Barlow JW, Funder JW. (1982) Steroid-induced vasoconstriction; glucocorticoid antagonist studies. *J Clin Endocrinol Metab* **54**: 1075-1077.

Martial JA, Baxter JD, Goodman HM, Seeburg PH. (1977) Regulation of growth hormone messenger RNA by thyroid and glucocorticoid hormones. *Proc Natl Acad Sci USA* **74**: 1816-1820.

Martin JB, Brazeau P, Tannenbaum GS, Willoughby JO, Epelbaum J, Terry LC, Durand D. (1978) Neuroendocrine organisation of growth hormone regulation. In "The Hypothalamus" (eds Reichlen S, Baldessarini RJ, Martin JB), pp 329, Raven Press, NY.

Martin CE, Cake MH, Hartmann PF, Cook IF. (1977) Relationship between foetal corticosteroids, maternal progesterone, and parturition in the rat. *Acta Endocrinol* **84**: 167-176.

Martin JB, Renaud LP, Brazeau P. (1974) Pulsatile growth hormone secretion: suppression by hypothalamic ventromedial lesions and by long-acting somatostatin. *Science* **186**: 538-540.

Marver D. (1984) Evidence of corticosteroid action along the nephron. *Am J Physiol* **246**: F111-F123.

McClellan PD, Linko P, Yeowell HN, Goldstein JA. (1989) Hormonal regulation of male-specific rat hepatic cytochrome P-450g (P-450IIC13) by androgens and the pituitary. *J Biol Chem* **264**: 18960-18965.

McEwen BS, Lambdin LT, Rainbow TC, Denicola AF. (1986a) Aldosterone effects on salt apetite in adrenalectomised rats. *Neuroendocrinol* **43**: 38-43.

McEwen BS, deKloet ER, Rostene W. (1986b) Adrenal steroid receptors and action in the nervous system. *Physiol Rev* **66**: 1121-1188.

McEwen BS. (1979) Influences of adrenocortical hormones on pituitary and brain function. In "Glucocorticoid Hormone Action" (eds Baxter JD, Rousseau GG), pp 467-492, Springer-Verlag, NY.

McKenna TJ, Island DP, Nicholson WE, Liddle GW. (1978) The effects of potassium on early and late steps in aldosterone biosynthesis in cells of the zona glomerulosa. *Endocrinol* **103**: 1411-1416.

Mercer W, Krozowski ZS. (1992) Localisation of an 11ß-hydroxysteroid dehydrogenase activity to the distal nephron. Evidence for the existence of two species of dehydrogenase in the rat kidney. *Endocrinol* **130**: 540-543.

Mercer W, Obeyesekere V, Smith R, Stuchbery S, Krozowski Z. (1991) Characterisation of 11ß-hydroxysteroid ehydrogenase gene expression; isolation of variant cDNA clones from a rat kidney library. In: Aldosterone: Fundamental apects (eds JP Bonvalet, N farman, M Lombes, ME Rafestin-Oblin), pp343, Paris: INSERM/John Libbey Eurotext Ltd.

Meyer WJ, Nichols NR. (1981) Mineralocorticoid binding in cultured smooth muscle cells and fibroblasts from rat aorta. *J Steroid Biochem* **14**: 1157-1168.

Michael AE, Gregory S, Walker SM, Antoniw JW, Shaw RW, Edwards CRW, Cooke BA. (1993) Ovarian 11ß-hydroxysteroid dehydrogenase:potential predictor of conception by in vitro fertilisation and embryo transfer. *Lancet* **342**: 711-712.

Miesfeld R, Godowski PJ, Maler BA, Yamamoto KR. (1987) Glucocorticoid receptor mutants that define a small region sufficient for enhancer activation. *Science* **236**: 423-427.

Miesfeld R, Okret S, Wikstrom AC, Yamamoto KR. (1984) Characterisation of a steroid hormone receptor gene and mRNA in wild type and mutant cells. *Nature* **312**: 779-781.

Millard WJ, Martin Jr JB, Audet J, Sagar SM, Martin JB. (1982) Evidence that reduced growth hormone secretion observed in monosodium glutamate-treated rats is the result of a deficiency in growth homone-releasing factor. *Endocrinol* **110**: 540-550.

Miner JN, Yamamoto KR. (1992) The basic region of AP-1 specifies glucocorticoid receptor activity at a composite response element. *Genes Dev* **6**: 2491-2501.

Miner JN, Yamamoto KR. (1991) Regulatory crosstalk at composite response elements. *Trends Biochem Sci* **16**: 423-426.

Mizobuchi M, Frohman MA, Downs TR, Frohman LA. (1991) Tissue-specific transcription initiation and effects of growth hormone (GH) deficiency on the regulation of mouse and rat GH-releasing hormone gene in hypothalamus and placenta. *Mol Endocrinol* **5**: 476-484.

Moisan M-P, Edwards CRW, Seckl JR. (1992a) Ontogeny of 11ß-hydroxysteroid dehydrogenase in rat brain and kidney. *Endocrinol* **130**: 400-404.

Moisan M-P, Edwards CRW, Seckl JR. (1992b) Differential promoter usage by the rat 11ß-hydroxysteroid dehydrogenase gene. *Mol Endocrinol* **6**: 400-404.

Moisan M-P, Seckl JR, Edwards CRW. (1990a) 11ß-hydroxysteroid dehydrogenase bioactivity and messenger RNA expression in rat forebrain: localization in hypothalamus, hippocampus and cortex. *Endocrinol* **127**: 1450-1455.

Moisan M-P, Seckl JR, Brett LP, Monder C, Agarwal AK, White PC, Edwards CRW. (1990b) 11beta-hydroxysteroid dehydrogenase messenger ribonucleic acid

expression, bioactivity and immunoreactivity in rat cerebellum. *J Neuroendocrinol* **2**: 853-858.

Moisan M-P, Seckl JR, Edwards CRW. (1990c) Effect of adrenalectomy and dexamethasone administration on 11ß-hydroxysteroid dehydrogenase in vitro activity in rat brain and kidney. *J Physiol* **434**: 87P.

Monder C, Lakshmi V, Miroff Y. (1991) Kinetic studies on rat liver 11ß-hydroxysteroid dehydrogenase. *Biochim Biophys Acta* **1115**: 23-29.

Monder C. (1991a) Corticosteroids, kidneys, sweet roots and dirty drugs. *Mol Cell Endocrinol* **78**: C95-C98.

Monder C. (1991b) Corticosteroids, receptors, and the organ-specific functions of 11ß-hydroxysteroid dehydrogenase. *FASEB J* **5**: 3047-3054.

Monder C, Lakshmi V. (1990) Corticosteroid 11ß-dehydrogenase of rat tissues: immunological studies. *Endocrinol* **126**: 2435-2443.

Monder C, Stewart PM, Lakshmi V, Valentino R, Burt D, Edwards CRW. (1989) Liquorice inhibits corticosteroid 11ß-dehydrogenase of rat kidney and liver: In vivo and in vitro studies. *Endocrinol* **125**: 1046-1053.

Monder C, Lakshmi V. (1989) Evidence for kinetically distinct forms of corticosteroid 11ß-dehydrogenase in rat liver microsomes. *J Steroid Biochem* **32**: 77-83.

Monder C, Shackleton CHL. (1984) 11ß-hydroxysteroid dehydrogenase: Fact or fancy? *Steroids* **44**: 383-417.

Moore CCD, Mellon SH, Murai J, Siiteri PK, Miller WL. (1993) Structure and function of the hepatic form of 11ß-hydroxysteroid dehydrogenase in the squirrel monkey, an animal model of glucocorticoid resistance. *Endocrinol* **133**: 368-375.

Morgan ET, Gustafsson J-A. (1987) Sex specific isozymes of P-450. *Steroids* **49**: 213-245.

Morgenstern JC, Land H. (1990) A series of mammalian expression vectors and characterisation of their expression of a reporter gene in stably and transiently transfected cells. *Nucl Acids Res* **18**: 1068.

Muller M, Renkawitz R. (1991) The glucocorticoid receptor. *Biochim Biophys Acta* **1088**: 171-182.

Munck A. (1962) Stidues on the mode of action of glucocorticoids in rats. II. The effects in vivo and in vitro on net glucose uptake by isolated adiopse tissue. *Biochim Biophys Acta* **57**: 318-326.

Murphy BEP. (1981) Demonstration of novel compounds in human fetal tissues and a consideration of their possible role in parturition. *Am J Obstet Gynecol* **139**: 353-358.

Murphy BEP. (1978) Cortisol production and inactivation by human lung during gestation and infancy. *J Clin Endocrinol Metab* **47**: 243-248.

Murphy BEP, Clark SJ, Donald IR, Pinsky M, Vedady D. (1974) Conversion of maternal cortisol to cortisone during placental transfer to the human fetus. *Am J Obstet Gynecol* **118**: 538-541.

Náray-Fejes-Tóth A. Watlington CO, Fejes-Tóth G. (1991) 11ß-hydroxysteroid dehydrogenase activity in the renal target cells of aldosterone. *Endocrinol* **129**: 17-21.

Naville D, Keeney DS, Jenkin G, Murry BA, Head JR, Mason JI. (1991) Regulation of expression of male-specific rat liver microsomal 3ß-hydroxysteroid dehydrogenase. *Mol Endocrinol* **5**: 1090-1100.

New MI, Levine L, Biglieri EG, Pareira J, Ulick S. (1977) Evidence for an unidentified steroid in a child with apparent mineralocorticoid hypertension. *J Clin Endocrinol Metab* **44**: 924-933.

Nicholas TE, Lugg MA. (1982) The physiological significance of 11ß-hydroxysteroid deydrogenase in the rat lung. *J Steroid Biochem* **17**: 113-118.

Nichols NR, Nguyen HH, Meyer WJ. (1985) Physical separation of aortic corticoid receptors with Type I and Type II specificities. *J Steroid Biochem* **22**: 577-582.

Nikkila H, Tannin GM, Taylor NF, Kalaitzoglou G, Monder C, White PC. (1993) Defects in the HSD11 gene encoding 11ß-hydroxysteroid dehydrogenase are not found in patients with apparent mineralocorticoid excess or 11-oxoreductase deficiency. *Endocrine Soc 75th Ann Meet Prog Abstr*: 410.

Nimrod A, Ryan KJ. (1975) Aromatization of androgens by human abdominal and breast fat tissue. *J Clin Endocrinol Metab* **40**: 367-372.

Nordeen SK. (1988) Luciferase reporter gene vectors for analysis of promoters and enhancers. *Biotech* **6**: 454-456.

Norstedt G, Palmiter R. (1984) Secretory rhythm of growth hormone regulates sexual differentiation of mouse liver. *Cell* **36**: 805-812.

Obeid J, Curnow KM, Aisenberg J, White PC. (1993) Transcripts originating in intron 1 of the HSD11 (11s-hydroxysteroid dehydrogenase) gene encode a truncated polypeptide that is enzymatically inactive. *Mol Endocrinol* **7**: 154-160.

Oberfield SE, Levine LS, Carey RM, Greig F, Ulick S, New MI. (1983) Metabolic and blood pressure responses to hydrocortisone in the syndrome of apparent mineralocorticoid excess. *J Clin Endocrinol Metab* **56**: 332-339.

Odom MJ, Snyder JM, Boggaram V, Mendelson CR. (1988) Glucocorticoid regulation of the major surfactant associated protein (SP-A) and its messenger nucleic acid and of morphological development of human fetal lung in vitro. *Endocrinol* **123**: 1712-1720.

O'Malley BW. (1990) The steroid hormone receptor superfamily: more excitement predicted for the future. *Mol Endocrinol* **4**: 363-369.

O'Malley BW, Conneely OM. (1992) Orphan receptors: a unifying hypothesis for activation. *Mol Endocrinol* **6**: 1359-1361.

Ono M, Miki N, Demura H. (1991) Effect of antiserum to rat growth hormone (GH)-releasing factor on physiological GH secretion in the female rat. *Endocrinol* **129**: 1791-1796.

Oskarsson J, Olofsson S-O, Bondjers G, Eden S. (1989) Differential effects of continuous versus intermittent administration of growth hormone to hypophysectomised female rats on serum lipoproteins and their apoproteins. *Endocrinol* **125**: 1638-1649.

Osinski PA. (1960) 11ß-ol dehydrogenase in human placenta. *Nature* **187**: 777-779.

Painson J-C, Thorner MO, Kreig RJ, Tannenbaum GS. (1992) Short term adult exposure to estradiol feminises the male pattern of spontaneous and growth hormone-releasing factor-stimulated growth hormone secretion in the rat. *Endocrinol* **130**: 511-519.

Painson JC, Tannenbaum GS. (1991) Sexual dimorphism of somatostatin and growth hormone-releasing factor signaling in the control of pulsatile growth hormone secretion in the rat. *Endocrinol* **128**: 2858-2866.

Parker MG. (1993) Steroid and related receptors. *Current Opinion Cell Biol* **5**: 499-504.

Pasqalini JR, Nguyen B-L, Uhlrich F, Wiquist N, Diczfalusy E. (1970) Cortisol and cortisone metabolism in the human feto-placental unit at midgestation. *J Steroid Biochem* **1**: 209-219.

Patel PD, Sherman TG, Goldman DJ, Watson SJ. (1989) Molecular cloning of a mineralocorticoid (type I) receptor complimentary DNA from rat hippocampus. *Mol Endocrinol* **3**: 1877-1885.

Patel YC. (1979) Growth hormone stimulates hypothalamic somatostatin. *Life Sci* **24**: 1589-1594.

Pearson CM, Wood FD. (1959) Studies of polyarthritis and other lesions in rats by injection of mycobacterial adjuvant. I. General clinical and pathological characteristics and some modifying factors. *Arthr Rheum* **2**: 440-459.

Pepe GJ, Waddel BJ, Stahl SJ, Albrecht ED. (1988) The regulation of transplacental cortisol-cortisone metabolism by estrogen in pregnant baboons. *Endocrinol* **122**: 78-83.

Pepe GJ, Albrecht ED. (1987) Fetal regulation of transplacental cortisol-cortisone metabolism in the baboon. *Endocrinol* **120**: 2529-2533.

Peskar K. (1976) Effect of carbenoxolone on parostaglandin synthesis and degradation. J Pharm Pharmacol 28: 146-148.

Peterson NA, Chaikoff IL, Jones C. (1965) The in vitro conversion of cortisol to cortisone by subcellular brain fractions of young and adult rats. *J Neurochem* **12**: 273-278.

Philipou G, Higgins BA. (1985) A new defect in the peripheral conversion of cortisone to cortisol. *J Steroid Biochem* **22**: 435-436.

Phillips D.M., Lakshmi V., Monder C. (1989). Corticosteroid 11ß-dehydrogenase in rat testis. *Endocrinol* **125**: 209-216.

Picard D, Yamamoto KR. (1987) Two signals mediate hormone-dependent nuclear localisation of the glucocorticoid receptor. *EMBO J* **6**: 3333-3340.

Provencher PH, Mercer WR, Funder JW, Krozowski ZS. (1992) Identification and characterisation of an NAD-dependent 11ß-HSD in pig kidney. *Endocrine Soc 74th Ann Meet Prog Abstr*: 305.128.

Ptashne M. (1988) How eukaryotic transcriptional activator work. *Nature* **335**: 683-689.

Ptashne M. (1986) Gene regulation by proteins acting nearby and at a distance. *Nature* **322**: 697-701.

Quinn SJ, Williams GH. (1988) Regulation of aldosterone secretion. *Ann Rev Physiol* **50**: 409-426.

Quirk SJ, Slattery J, Funder JW. (1990a) 11ß-hydroxysteroid dehydrogenase activity in the mammary gland. *J Steroid Biochem Mol Biol* **35**: 623-625.

Quirk SJ, Slattery JA, Funder JW. (1990b) Epithelial and adipose cells isolated from mammary glands of pregnant and lactating rats differ in 11ß-hydroxysteroid dehydrogenase activity. *J Steroid Biochem Mol Biol* **37**: 529-534.

Quirk SJ, Gannell JE, Fullerton MJ, Funder JW. (1988) Specificity and mechanism of biphasic action of glucocorticoids on α-lactalbumin production by rat mammary gland explants. *Endocrinol* **118**: 909-914.

Reinehart JJ, Wuest D, Ackerman GA. (1982) Corticosteroid alteration of human monocyte to macrophage differentiation. *J Immunol* **129**: 1436-1440.

Reinisch JM, Simon NG, Karwo WG et al. (1978) Prenatal exposure to prednisone in humans and animals retards intra-uterine growth. *Science* **202**: 436-438.

Reul JMHM, deKloet ER. (1986) Anotomical resolution of two types of corticoisteroid sites in rat brain with *in vivo* autoradiography and computerised image analysis. *J Steroid Biochem* **24**: 269-272.

Rivier J, Speiss J, Thorner M, Vale W. (1982) Characterisation of a growth hormone-releasing factor from a human pancreatic islet tumour. *Nature* **300**: 276-278.

Robinson J, Canavan P, El Hah AJ, Goldspink DF. (1988) Maternal diabetes in rats I: effects on placental growth and protein turnover. *Diabetes* **37**: 1665-1670.

Rundle SE, Funder JW, Lakshmi V, Monder C. (1989) The intrarenal localisation of mineralocorticoid receptors and 11ß-dehydrogenase:immunocytochemical studies. *Endocrinol* **125**, 1700-1704.

Rusvai E, Naray-Fejes-Toth A. (1993) A new isoform of 11ß-hydroxysteroid dehydrogenase in aldosterone target cells. *J Biol Chem* **268**: 10717-10720.

Ryan DE, Lida S, Wood AW, Thomas PE, Lieber CS, Levin W. (1984) Characterisation of three highly purified cytochrome P-450s from hepatic microsomes of adult male rats. *J Biol Chem* **259**: 1239-1250.

Saez JM, Morera AM, Haour F, Evain D. (1977) Effects of in vivo administration of dexamethasone, corticotropin and human chronic gonadotropin on steroidogenesis and protein and DNA synthesis of testicular interstitial cells in prepubertal rats. *Endocrinol* **101**: 1256-1263.

Sakai RR, Lakshmi V, Monder C, McEwen BS. (1992) Immunocytochemical localisation of 11beta-hydroxysteroid dehydrogenase in hippocampus and other brain regions of the rat. *J Neuroendocrinol* **4**: 101-106.

Salassa RM, Mattox VR, Rosevear JW. (1962) Inhibition of mineralocorticoidactivity of liquorice by spironolactone. *J Clin Endocrinol* **22**:1156-1159.

Sambrook J, Fritsch EF, Maniatis T. (1989) Molecular cloning: a laboratory manual. 2nd edition Cold Spring Harbour Laboratory Press, Cold Spring Harbour NY.

Sapolsky RM, Krey LC, McEwen BS. (1986) The neuroendocrinology of stress and ageing. The glucocorticoid cascade hypothesis. *Endocrine Rev* **7**: 284-301.

Sapolsky RM, Krey LC, McEwen BS. (1985) Prolonged glucocorticoid exposure reduces hippocampal neuron number: implications for aging. *J Neurosci* **5**: 1222-1227.

Sapolsky RM, Pulsinelli W. (1985) Glucocorticoids potentiate ischemic injury to neurones: therapeutic implications. *Science* **229**: 1397-1400.

Sarlis NJ, Chowdrey HS, Stephanou A, Lightman SL. (1992) Chronic activation of the hypothalamo-pituitary-adrenal axis and loss of circadian rhythm during adjuvant-induced arthritis in the rat. *Endocrinol* **130**: 1775-1779.

Saruta T, Suzuki H, Manda M, Igarashi Y, Kondo K, Senba S. (1986) Multiple factors contribute to the pathogenesis of hypertension in Cushing's syndrome. *J Clin Endocrinol Metab* **62**: 275-279.

Saunders A, Terry LC, Audet J, Brazeau P, Martin JB. (1976) Dynamic studies of growth hormone and prolactin secretion in the female rat. *Neuroendocrinol* **21**: 193-203.

Schreiber JR, Nakamura K, Erickson GF. (1982) Rat ovary glucocorticoid receptor: identification and characterisation. *Steroids* **71**: 986-989.

Schwartz J, Keil LC, Maselli J, Reid IA. (1983) Role of vasopressin in blood pressure regulation during adrenal insufficiency. *Endocrinol* **112**: 234-238.

Seckl JR, French KL, O'Donnell D, Meaney MJ, Nair NPV, Yates CM, Fink G. (1993) Glucocorticoid receptor gene expression is unaltered in hippocampal neurons in Alzheimer's disease. *Mol. Brain Res* **18**: 239-245.

Seckl JR, Kelly PAT, Sharkey J. (1991) Glycyrrhetinic acid, an inhibitor of 11ß-hydroxysteroid dehydrogenase, alters local cerebral glucose utilisation in vivo. *J Steroid Biochem Mol Biol* **39**: 777-779.

Seidman CE, Bloch KD, Klein KA, Smith JA, Seidman JG. (1984) Nucleotide sequence of the human and mouse atrial natriuretic factor genes. *Science* **226**: 1206-1209.

Shackleton CHL, Stewart PM. (1990) The hypertension of apparent mineralocorticoid excess syndrome. In: Endocrine Hypertension (eds EG Biglieri & JC Melby), pp 155-173, Raven Press, New York.

Sheppard K, Funder JW. (1987) Type I receptors in parotid, colon, and pituitary are aldosterone selective in vivo. Am J Physiol 253: E467-E471.

Shirazu K, Stumpf WE, Sar M. (1990) Evidence for direct action of oestradiol on growth hormone-releasing factor (GRF) in rat hypothalamus: localization of $^{3}$H-oestradiol in GRF neurones. *Endocrinol* **127**: 344-349.

Siebe H, Baude G, Lichtenstein I, Wang D, Buhler H, Hoyer GA, Hierholzer K. (1993) Metabolism of dexamethasone: Sites and activity in mammalian tissues. *Renal Physiol Biochem* **16**: 79-88.

Simpson ER, Waterman MR. (1988) Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. *Ann Rev Physiol* **50**: 427-440.

Slessor A. (1951) Studies concerning the mechanism of water retention in Addison's disease and in hypopituitarism. *J Clin Endocrinol Metab* **11**:700-723.

Sloviter RS, Valiquette G, Abrams GM, Ronk EC, Sollas AL, Paul LA, Neubort S. (1989) Selective loss of hippocampal granule cells in the mature rat brain after adrenalectomy. *Science* **243**: 535-538.

Smith BT, Torday JS, Giroud CJP. (1973) The growth promoting effect of cortisol on human fetal lung cells. *Steroids* **22**: 515-524.

Smith RE, Mercer WR, Provencher PH, Obeyeskere V, Krozowski ZS. (1992) The heterogeneity of 11ß-hydroxysteroid dehydrogenase activities in the rat hippocampus implies complex regulation of steroid hormone action. *Clin Experiment Pharmacol Physiol* **19**: 365-368.

Smith RE, Funder JW. (1991) Renal 11ß-hydroxysteroid dehydrogenase activity: Effects of age, sex and altered hormonal status. *J Steroid Biochem Mol Biol* **38**: 265-267.

Solomon J, Greep RO. (1959) The effect of alterations in thyroid function on the pituitary growth hormone content and acidophil cytology. *Endocrinol* **65**: 158-164.

Song GW, Lorenzo B, Reidenberg MM. (1991) Inhibitory effects of gossypol on corticosteroid 11ß-hydroxysteroid dehydrogenase from guinea pig kidney: A possible mechanism for causing hypokalaemia. *J Steroid Biochem Mol Biol* **39**: 169-176.

Souness GW, Morris DJ. (1989) The antinatriuretic and kalliuretic effects of the glucocorticoids corticosterone and cortisol following pretreatment with carbenoxolone sodium (a liquorice derivative) in the adrenalectomised rat. *Endocrinol* **124**: 1588-1590.

Sousa RJ, Tannery NH, Lafer EM. (1989) In situ hybridisation mapping of glucocorticoid receptor messenger ribonucleic acid in the rat brain. *Mol Endocrinol* **3**: 481-494.

Stachura ME. (1976) Influence of synthetic somatostatin upon growth hormone release from perfused rat pituitaries. *Endocrinol* **99**: 678-683.

Stewart PM, Wallace AM, Atherden SM, Shearing CH, Edwards CRW. (1990) Mineralocorticoid activity of carbenoxolone: contrasting effects of carbenoxolone and liquorice on 11ß-hydroxysteroid dehydrogenase activity in man. *Clin Sci* **78**: 49-54.

Stewart PM, Corrie JET, Shackleton CHL, Edwards CRW. (1988) The syndrome of apparent mineralocorticoid excess: a defect in the cortisol-cortisone shuttle. *J Clin Invest* **82**: 340-349.

Stewart PM, Wallace AM, Valentino R, Burt D, Shackleton CHL, Edwards CRW. (1987) Mineralocorticoid activity of liquorice: 11ß-hydroxysteroid dehydrogenase deficiency comes of age. *Lancet* **1**: 821-823.

Strom A, Mode A, Zaphiropoulos P, Nilsson A-G, Morgan E, Gustafsson J-A. (1988) Cloning and pretranslational hormonal regulation of testosterone 16α-hydroxylase ($P450_{16\alpha}$) in male rat liver. *Acta Endocrinol* **118**: 314-320.

Suzuki M, Takeuchi A, Kakegawa T. (1980) Control of rat growth hormone secretion by thyroid hormone. Sixth International Congress Endocrinology, p298 (abstract).

Tannin GM, Agarwal AK, Monder C, New MI, White PC. (1991) The human gene for 11-beta-hydroxysteroid dehydrogenase. *J Biol Chem* **266**: 16653-8.

Tannenbaum GS, Eikelboom R, Ling N. (1983) Human pancreas GH-releasing factor analog restores high amplitude GH pulses in CNS lesion-induced GH deficiency. *Endocrinol* **113**: 1173-1175.

Tannenbaum GS. (1981) Growth hormone secretory dynamics in streptozotocin diabetes: evidence of a role for endogenous circulating somatostatin. *Endocrinol* **108**: 76-82.

Tannenbaum GS. (1980) Evidence for autoregulation of growth hormone secretion via the central nervous system. *Endocrinol* **107**: 2117-2120.

Tannenbaum GS, Epelbaum J, Colle E, Brazeau P, Martin JB. (1978) Antiserum to somatostatin reverses starvation-induced inhibition of growth hormone but not insulin secretion. *Endocrinol* **102**: 1909-1914.

Tannenbaum GS, Martin JB. (1976) Evidence for endogenous ultradian rhythm governing growth hormone secretion in the rat. *Endocrinol* **98**: 562-570.

Taylor NF, Bartlett WA, Dawson DJ, Enoch BA. (1984) Corticosne reductase deficiency: Evidence for a new inborn error of metabolism of adrenal steroids. *J Endocrinol* (Suppl) **102**: Abstract 50.

Teelucksingh S, Mackie A, Burt D, McIntyre M, Brett L, Edwards CRW. (1990) Potentiation of hydrocortisone activity in skin by glycyrrhetinic acid. *Lancet* **335**: 1060-1063.

Tempel DL, Liebowitz SF. (1989) PVN steroid implants: Effect on feeding patterns and macronutrient selection. *Brain Res Bull* **23**: 553-560.

Terry LC, Martin JB. (1981) The effects of lateral hypothalamic-medial forebrain stimulation and somatostatin antiserum on pulsatile growth hormone secretion in freely behaving rats: evidence for a dual regulatory mechanism. *Endocrinol* **109**: 622-627.

The VL, Labrie C, Zhao HF, Couet J, Lachance Y, simard J, Leblanc G, Cote J, Berube D, Gagne R, Labrie F. (1989) Characterisation of cDNAs for human estradiol 17ß-dehydrogenase and assignment of the gene to chromosome 17: Evidence of two mRNA species with distinct 5' termini in human placenta. *Mol Endocrinol* **3**: 1301-1309.

Tollet P, Enberg B, Mode A. (1990) Growth hormone regulation of cytochrome P-450IIC12, insulin-like growth factor I and growth hormone receptor mRNA expression in primary rat hepatocytes: a hormonal interplay with insulin, IGF-I and thyroid hormone. *Mol Endocrinol* **4**: 1934-1942.

Torday JS, Olson EB Jr, First NL. (1976) Production of cortisol from cortisone by the isolated perfused fetal rabbit lung. *Steroids* **27**: 869-880.

Ueda H, Sun G-C, Murata T, Hirose S. (1992) A novel DNA binding motif abuts the zinc finger domain of insect nuclear hormone receptor FTX-F1 and mouse embryonal long terminal repeat-binding protein. *Mol Cell Biol* **12**: 5667-5672.

Uht RM, McKelvy JF, Harrison RW, et al. (1988) Demonstration of glucocorticoid receptor-like immunoreactivity in glucocorticoid-sensitive vasopressin and corticotropin-releasing factor neurons in the hypothalamic paraventricular nucleus. *J Neurosci Res* **19**: 405-411.

Ulick S, Levine LS, Gunczler P, Zanconato G, Ramirez LC, Raub W, Rosler A, Bradlow HL. (1979) A syndrome of apparent mineralocorticoid excess associated with defects in the peripheral metabolism of cortisol. *J Clin Endocrinol Metab* **49**: 757-764.

Ulick S, Ramirez LC, New MI. (1977) An abnormality in steroid reductive metabolism in a hypertensive syndrome. J *Clin Endorinol Metab* **44**: 799-802.

Ulmann A, Menard J, Corvol P. (1975) Binding of glycyrrhetinic acid to kidney mineralocorticoid receptors. *Endocrinol* **97**: 46-51.

Umesono K, Murakami KK, Thompson CC, Evans RM. (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. *Cell* **65**: 1255-1266.

Van Den Berg DTWM, de Kloet ER, Van Dijken HH, DeJong W. (1990) Differential central effects of mineralocorticoid and glucocorticoid agonist and antagonists on blood pressure. *Endocrinol* **126**: 118-124.

Vanderbilt JN, Miesfeld R, Maler BA, Yamamoto KR. (1987) Intracellular receptor concentration limits glucocorticoid-dependent enhancer activity. *Mol Endocrinol* **1**: 68-74.

Van Eekelen JAM, Jiang W, de Kloet ER, Bohn MC. (1989) Distribution of the mineralocorticoid and glucocorticoid receptor mRNAs in the rat hippocampus. *J Neurosci Res* **21**: 88-94.

Wajchenberg BL, Pereira VG, Kieffer J, Ursic S. (1969) Effect of dexamethasone on calcium metabolism and $^{47}Ca$ kinetics in normal subjects. *Acta Endocrinol* **61**: 173-192.

Walker BR, Williams BC, Edwards CRW. (1994) Regulation of 11ß-hydroxysteroid dehydrogenase activity by the hypothalamic-pituitary-adrenal axis in the rat. *J Endocrinol* (in press).

Walker BR, Campbell JC, Williams BC, Edwards CRW. (1992) Tissue-specific distribution of the $NAD^+$-dependent isoform of 11ß-hydroxysteroid dehydrogenase. *Endocrinol* **131**, 970-972.

Walker BR, Yau JLW, Brett LP, Seckl JR, Monder C, Williams BC, Edwards CRW. (1991) 11ß-hydrioxysteroid dehydrogenase in vascular smooth muscle and heart: Implications for cardiovascular responses to glucocorticoids. *Endocrinol* **129**: 3305-3312.

Walters MR. (1985) Steroid hormone receptors and the nucleus. *Encorine Rev* **6**: 512-543..

Watabe T, Tanaka K, Kumagae M, Itoh S, Hasegawa M, Horuchi T, Miyabe S, Ohno H, Shimizu N. (1987) Diurnal rhythm of plasma immunoreactive corticotropin-releasing factor in normal subjects. *Life Sci* **40**: 1651-1655.

Webb CB, Szabo M, Frohman LA. (1983) Ectopic growth homone-releasing factor and dibutyryl cyclic adenosine monophosphate-stimulated growth hormone release in vitro: effects of corticosterone and estradiol. *Endocrinol* **113**: 1191-1196.

Wehling M, Armanini D, Strasser T, Weber PC. (1987) Effect of aldosterone on sodium and potassium concentrations in human mononuclear leukocytes. *Am J Physiol* **252**: E505-E508.

Wehrenberg WB, Guistina A. (1992) Basic Counterpoint: Mechanisms and pathways of gonadal steroid modulation of growth hormone secretion. *Endocrine Rev* **13**: 299-308.

Wehrenberg WB, Baird A, Ling N. (1983) Potent interaction between glucocorticoids and growth hormone-releasing factor in vivo. *Science* **221**: 556-558.

Wehrenberg WB, Brazeau P, Luben R, Bohlen P, Guillemin R. (1982) Inhibition of the pulsatile secretion of growth hormone by monoclonal antibodies to the hypothalamic growth hormone releasing factor (GRF). *Endocrinol* **111**: 2147-2152.

Weinberger C, Thompson CC, Ong ES, Lebo R, Groul DJ, Evans RM. (1986) The c-erbA gene encodes a thyroid hormone receptor. *Nature* **324**: 641-646.

Welsh JR, Bambino TH, Hseuh AJW. (1982) Mechanism of glucocorticoid-induced suppression of testicular androgen biosynthesis in vitro. *Biol Repro* **27**: 1138-1146.

Werder E, Zachman M, Vollmin J, Verrat R, Prader A. (1974) Unusual steroid excretion in a child with low renin hypertension. *Res Steroids* **6**: 385-389.

Werner H, Koch Y, Baldino F, Gozes I. (1988) Steroid regulation of somatostatin mRNA in the rat hypothalamus. *J Biol Chem* **263**: 7666-7671.

Whincup PH, Cook DG, Shaper AG. (1989) Early influences on blood pressure: A study of children aged 5-7 years. *Br Med J* **299**: 587-591.

Whorwood CB, Franklyn JA, Sheppard MC, Stewart PM. (1992) Tissue-localisation of 11ß-hydroxysteroid dehydrogenase and its relationship to the glucocorticoid receptor. J*Steroid Biochem Molecular Biol* **41**, 21-28.

Widmaier EP, Plotsky PM, Sutton SW, (1988) Regulation of corticotropin-releasing factor secretion in vitro by glucose. *Am J Physiol* **255**: E287-E292.

Williams LJS, Napolitano A, Rajan V, Edwards CRW, Seckl JR, Chapman KE. (1993) C/EBP - a putative transcriptional regulator of 11ß-OHSD. *J Endocrinol* **139**: (suppl) 07 (Abstract).

Willoughby JO, Menadue M, Zeegers P, Wise PH, Oliver JR. (1980) Effects of human growth hormone on the secretion of rat growth hormone. *J Endocrinol* **86**: 165-169.

Willoughby JO, Martin JB. (1978) The suprachiasmatic nucleus synchronises growth hormone secretory rhythms with the light-dark cycle. *Brain Res* **151**: 413-417.

Wilson TE, Fahrner TJ, Johnston M, Milbrandt J. (1991) Identification of the DNA binding site for NGFI-B by genetic selection in yeast. *Science* **252**: 1296-1390.

Yamamoto KR. (1985) Steroid receptor regulated transcription of specific genes and gene networks. *Ann Rev Genet* **19**: 209-252.

Yang K, Smith CL, Dales D, Hammond GL, Challis JR. (1992) Cloning of an ovine 11 beta-hydroxysteroid dehydrogenase complementary deoxyribonucleic acid: tissue and temporal distribution of its messenger ribonucleic acid during fetal and neonatal development. *Endocrinol* **131**: 2120-2126.

Yau JLW, van Haarst AD, Moisan M-P, Fleming S, Edwards CRW, Seckl JR. (1991) 11ß-hydroxysteroid dehydrogenase mRNA expression in rat kidney. *Am J Physiol* **260**: F764-F767.

Yu VC, Delsert C, Andersen B, Holloway JM, Devary OV, Naar AM, Kim SY, Boutin J-M, Glass CK, Rosenfeld MG. (1991) RXRß: a coregulator that enhances binding of retinoic acid, thyroid hormone and vitamin D receptors to their cognate response elements. *Cell* **67**: 1251-1266.

Yu DTY, Clements PJ, Paulus HE, Peter JB, Levy J, Barnett EV. (1974) Human lymphocyte subpopulations. Effects of corticosteroids. *J Clin Invest* **53**: 565-571.

Zeitler P, Argente J, Chowen-Breed JA, Clifton DK, Steiner RA. (1990) Growth hormone-releasing hormone messenger ribonucleic acid in the hypothalamus of the adult male rat is increased by testosterone. *Endocrinol* **127**: 1362-1368.

Zhang X-K, Hoffmann B, Tran PB-V, Graupner G, Pfahl M. (1992) Retinoid X receptor is an auxilliary protein for thyroid hormone and retinoic acid receptors. *Nature* **355**: 441-446.

Zorilla R, Simard J, Rheaume E, Labrie F, Pelletier G. (1990) Multihormonal control of pre-pro-somatostatin mRNA levels in the periventricular nucleus of the male and female rat hypothalamus. *Neuroendocrinol* **52**: 527-536.

Zumoff B, Bradlow HL, Levin J, Fukushima DK. (1983) Influence of thyroid function on the in vivo cortisol-cortisone equilibrium in man. *J Steroid Biochem* **18**: 437-440.

Zweifach BW, Schorr E, Black MM. (1953) The influence of the adrenal cortex on the terminal vascular bed. *Ann NY Acad Sci* **56**: 626-633.

**OMITTED REFERENCES**

Arnold J. (1866) Ein beitrag zu der feineren structur und dem chemismus der nebennieren. *Arch Pathol Anat Physiol Klin Med* **35**: 64-107.

Morris DJ. (1981) The metabolism and mechanism of action of aldosterone. *Endocrine Rev* **2**: 234-247.

Orth DN, Kovacs WJ, Debold CR. (1991) In "Williams Textbook of Endocrinology" (eds Wilson JD, Foster DW 8th Edition), pp 489-620, WD Saunders Company, Philadelphia, PA.

# 11β-Hydroxysteroid dehydrogenase in the rat ovary: high expression in the oocyte

**R. Benediktsson, J. L. W. Yau, S. Low, L. P. Brett*, B. E. Cooke†, C. R. W. Edwards** and **J. R. Seckl**

Departments of Medicine and *Pathology, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU, U.K.

†Department of Biochemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, U.K.

(Requests for offprints should be addressed to J. R. Seckl)

RECEIVED 24 January 1992

## ABSTRACT

The enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) catalyses the conversion of physiological glucocorticoids to inactive products, thus modifying the access of glucocorticoids to glucocorticoid and mineralocorticoid receptors. Glucocorticoids may affect ovarian function both indirectly and via binding to ovarian receptors. We have demonstrated 11β-HSD bioactivity and mRNA expression in rat ovary *in vitro*. The enzyme was localized to oocytes and luteal bodies immunohistochemically using two antibodies raised against purified rat liver 11β-HSD. These data are supported by in-situ hybridization studies, which also localized 11β-HSD mRNA expression to oocytes and luteal bodies. The results suggest that 11β-HSD may modulate the effects of glucocorticoid on ovarian function.

*Journal of Endocrinology* (1992) **135,** 53–58

## INTRODUCTION

Glucocorticoid hormones are important regulators of cell function in many tissues. Recent evidence suggests that glucocorticoids affect ovarian physiology. Thus systemic administration of exogenous glucocorticoids has been shown to reduce ovarian weight (Inazu, Iwata & Satoh, 1990) and to inhibit ovulation (Baldwin & Sawyer, 1974). Although the inhibition of ovulation is thought to reflect glucocorticoid actions at the anterior pituitary, where they inhibit the preovulatory luteinizing hormone (LH) surge (Baldwin & Sawyer, 1974), the presence of glucocorticoid receptors (Schreiber, Nakamura & Erickson, 1982) and glucocorticoid-responsive gene products (Malbon & Hadcock, 1988; Albiston, Lock, Burger & Krozowski, 1990) in the ovary suggests that direct effects also occur. In support of this, glucocorticoids have been shown to inhibit follicle-stimulating hormone (FSH)-stimulated aromatase activity (Hsueh & Erickson, 1978) and to augment progesterone accumulation (Adashi, Jones & Hsueh, 1981) in granulosa cells *in vitro*. Similarly, glucocorticoids stimulate the production of plasminogen activator by isolated granulosa cells (Wang & Leung, 1989; Jia & Hsueh, 1990), an action thought to be important in the control of ovulation (Reich, Miskin & Tsafriri, 1985).

Both protein purification and more recent molecular cloning studies have demonstrated the existence of two types of glucocorticoid receptor, type-I (high-affinity or mineralocorticoid receptor) and type-II (low-affinity or glucocorticoid receptor). Although the type-I and -II receptors show well-described hierarchies of affinity for the various physiological and synthetic glucocorticoids *in vitro*, ligand access to receptors in many tissues *in vivo* is regulated by 11β-hydroxysteroid dehydrogenase (11β-HSD). This microsomal enzyme catalyses the reversible conversion of physiological glucocorticoids (cortisol in man, corticosterone in the rat) to inactive products (cortisone or 11-dehydrocorticosterone). The 11-dehydro products have very low affinities for either type-I or type-II receptors. The activity of 11β-HSD appears to be responsible for the in-vivo aldosterone selectivity of the otherwise non-selective renal type-I receptor (Edwards, Stewart, Burt *et al.* 1988; Funder, Pearce, Smith & Smith, 1988) and modulates glucocorticoid access to type-II receptors in the skin (Teelucksingh,

 
0022-0795/92/0135-0053 $02.00/0

Mackie, Burt *et al.* 1990) and cerebellum (Moisan, Seckl, Brett *et al.* 1990*a*). Within reproductive tissues, 11β-HSD is present in the placenta where it is thought to protect the fetus from the deleterious effects of exposure to maternal glucocorticoids (Beitins, Bayard, Ances *et al.* 1973; López Bernal, Anderson & Turnbull, 1982) and the enzyme has been demonstrated in the testis where it may regulate Leydig cell–glucocorticoid interactions which are critical in the control of testosterone production and the onset of puberty (Phillips, Lakshmi & Monder, 1989).

By analogy with the testis, the ovary might be expected to contain 11β-HSD which could regulate glucocorticoid access to receptors and thus modulate corticosteroid effects *in vivo*. Although previous studies have not found 11β-HSD bioactivity in the rat ovary (Ghraf, Vetter, Zandveld & Schriefers, 1975), human ovaries have been shown to be able to convert cortisol to cortisone (Murphy, 1981*a*), and to contain mRNA encoding 11β-HSD (Tannin, Agarwal, Monder *et al.* 1991). Therefore, we have re-examined whether 11β-HSD bioactivity and mRNA expression are present in the ovary of the rat, and have determined their tissue localization.

## MATERIALS AND METHODS

### 11β-HSD bioassay

*Dehydrogenase component*

Ovaries, obtained from adult female Wistar rats (240 g; $n = 3$–4) in pro-oestrus, were homogenized in Krebs–Ringer bicarbonate buffer (118 mmol NaCl/l, 3·8 mmol KCl/l, 1·19 mmol $KH_2PO_4$/l, 2·54 mmol $CaCl_2 \cdot 2H_2O$/l, 1·19 mmol $MgSO_4 \cdot 7H_2O$/l, 25 mmol $NaHCO_3$/l) using a Dounce tissue grinder. The total protein content was estimated colorimetrically (Bio-Rad protein assay kit, Hemel Hempstead, Herts, U.K.), using a sample of the homogenate. The incubation was carried out in duplicate with 0·0625 g, 0·25 g and 0·5 g protein/l and a final concentration of 200 μmol NAD/l and 12 nmol [1,2,6,7-$^3$H]corticosterone/l (specific activity: 84 Ci/mmol; Amersham International plc, Little Chalfont, Bucks, U.K.) in Krebs–Ringer buffer (+0·2% bovine serum albumin and glucose) for 10, 20 and 60 min at 37 °C. The total volume was 250 μl. Rat kidney, known to have very high 11β-HSD bioactivity, was used as a positive control (at 0·25 g protein/l for 10 min), whilst incubation of buffer alone provided an assay blank. After incubation, steroids were extracted with ethyl acetate and separated by high-pressure liquid chromatography (HPLC). The percentage conversion of [$^3$H]corticosterone to [$^3$H]11-dehydrocorticosterone was calculated from the radioactivity of each fraction. Results are expressed as means ± S.E.M.

*Reductase component*

For preparation of [$^3$H]11-dehydrocorticosterone, [$^3$H]corticosterone (Amersham) was incubated with rat kidney homogenate for 2 h at 37 °C in the presence of 200 μmol NAD/l. After incubation, the steroids were extracted with ethyl acetate and separated by thin-layer chromatography. Extraction yield was 42% and purity > 93% as assessed by HPLC. Ovarian homogenate was prepared as above ($n = 3$), and 0·25 g protein/l incubated with 12 nmol [$^3$H]11-dehydrocorticosterone/l for 10 min at 37 °C in the presence of 200 μmol NADH/l in Krebs–Ringer buffer as above. Steroids were separated and assayed by HPLC as for dehydrogenase activity.

### Northern analysis

Ovary, hippocampus, kidney and liver were rapidly removed from female Wistar rats (240 g) after cervical dislocation, snap-frozen and stored at −85 °C. Total RNA was extracted from each tissue by the acid guanidinium thiocyanate method, as previously described (Chomczynski & Sacchi, 1987). Approximately 20 μg ovary, 15 μg hippocampus and 10 μg kidney and liver total RNA were fractionated on a 1·2% agarose–0·7 mol formaldehyde gel/l and blotted on to nitrocellulose (Hybond C extra; Amersham International plc) by capillary transfer overnight. Hybridization was performed at 42 °C overnight in 50% formamide with a randomly primed $^{32}$P-labelled 11β-HSD cDNA probe cloned from a rat liver cDNA library (Agarwal, Monder, Eckstein & White, 1989), consisting of the excised p11DH insert. The membrane was washed to a final stringency of 0·2 × SSC (1 × SSC equals 0·5 mol NaCl and 0·015 mol sodium citrate per litre), 0·1% sodium dodecyl sulphate at 60 °C and exposed to Kodak XAR film for 2 days as previously described (Moisan, Seckl & Edwards, 1990*b*).

### Immunohistochemistry

Adult Wistar rats (240 g) were deeply anaesthetized with pentobarbitone and perfused through the ascending aorta with 250 ml ice-cold saline followed by 500 ml ice-cold paraformaldehyde (4 mol/l) in phosphate buffer (0·1 mol/l, pH 7·4). Ovaries were removed and post-fixed overnight. As previously described (Moisan *et al.* 1990*a*) sections (4 μm) were cut and immunostained using two separate polyclonal rabbit antisera (5–125 at 1:50 dilution and 5–126 at 1:100 dilution) raised against purified rat liver 11β-HSD as described elsewhere (Monder & Lakshmi, 1990). Detection was by the peroxidase–antiperoxidase method (Sternberger, Hardy, Cuculis & Meyer, 1970) using reagents from Dako Ltd, High Wycombe, Bucks, U.K. Control sections were immunostained using preimmune rabbit serum.

### In-situ hybridization

Adult Wistar rats (240 g) were killed by cervical dislocation, the ovaries rapidly removed and immediately frozen on dry ice. Cryostat sections (10 μm) were mounted on to gelatin- and poly-L-lysine-coated microscope slides and stored at −85 °C. Tissue sections were post-fixed in 4% paraformaldehyde/phosphate (0·1 mol/l) buffer and washed in three changes of 2 × SSC containing 0·02% diethylpyrocarbonate. T3 RAN polymerase (Promega Ltd, Southampton, Hants, U.K.) was used to transcribe a 598 bp antisense cRNA probe containing $^{35}$S-labelled UTP from Sty I-linearized pBluescript vector containing the 1265 bp 11β-HSD cDNA insert (Agarwal *et al.* 1989). The probe was denatured and added at a final concentration of $10 \times 10^6$ c.p.m./ml to hybridization buffer (50% formamide, 0·6 mol NaCl/l, 10 mmol Tris/l, pH 7·5, 0·02% Ficoll, 0·02% polyvinylpyrrolidone, 0·1% bovine serum albumin, 1 mmol EDTA/l, 0·1 g salmon sperm DNA/l, 0·1 g yeast tRNA/l, 100 g dextran sulphate/l and 10 mmol dithiothreitol/l) as previously described (Yau, Van Haarst, Moisan *et al.* 1991). In brief, 65 μl aliquots of probe were pipetted on to sections and covered with a coverslip (22 × 50 mm) which was sealed with DPX mounting medium. Slides were incubated overnight at 50 °C. After hybridization, coverslips were removed by rinsing three to four times in 2 × SSC at room temperature, sections treated with RNAase A (30 g/l, 45 min at 37 °C) (Boehringer Mannheim, Lewes, East Sussex, U.K.) and washed in reducing salt concentrations to a final stringency of 0·1 × SSC/14 mmol 2-mercaptoethanol/l at 60 °C. After dehydration in increasing concentrations of ethanol in 0·3 mol sodium acetate/l, slides were dried in air, dipped in photographic emulsion (Ilford Mobberley, Knutsford, Cheshire, U.K.) and exposed in light-tight boxes at 4 °C for 21 days before being developed (D19, Ilford) and counterstained with haematoxylin–eosin. Controls were hybridized with non-complementary 'sense' probes of the same specific activity under identical conditions.

## RESULTS

### 11β-HSD activity

11β-HSD activity in the dehydrogenase direction was found in all ovarian homogenates and increased linearly with protein concentration up to 0·5 g/l (data not shown). The time curves for the three different protein concentrations (Text-fig. 1) indicate linearity with respect to time for up to 10 min. At a protein concentration of 0·25 g/l incubated for 10 min, ovarian activity was 10% of kidney activity (ovary, $n=3$: 179·6 ± 30·5 fmol product formed; kidney, $n=2$: 1742·4 ± 44·1 fmol product formed). No conversion of [$^3$H]11-dehydrocorticosterone to [$^3$H]corticosterone was found.

### Northern blots

A single band hybridizing to the 11β-HSD probe was detected in ovarian total RNA which corresponded to the major 11β-HSD mRNA species demonstrated in rat liver and hippocampus. There was no evidence of expression of multiple 11β-HSD mRNA species as found in rat kidney (Text-fig. 2).

### Immunohistochemistry

Using either antiserum to purified rat liver 11β-HSD, a consistently strong positive immunostaining was found in the oocyte (Pl. 1). Additionally, positive immunostaining of moderate or low intensity was detected in cells comprising the luteal bodies (not shown). This varied between the luteal masses rather than within a given luteal body, presumably reflecting their varying stages of maturation. No staining was observed in the granulosa cells, theca cells, stroma or other ovarian components (Pl. 1). No staining of any ovarian subregion was demonstrated with preimmune serum.

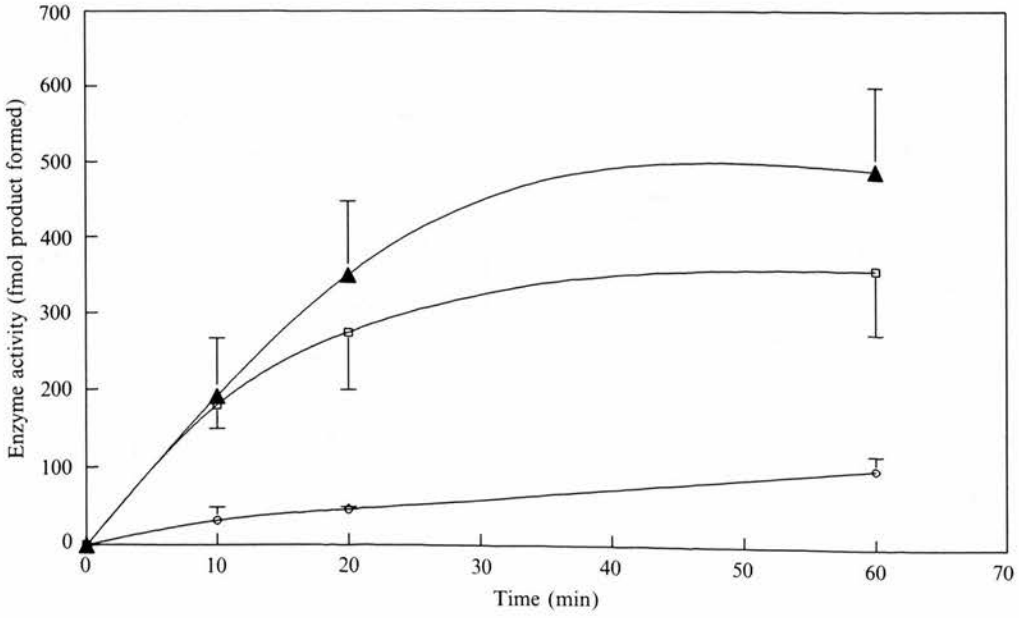
### In-situ hybridization

11β-HSD mRNA expression was localized to oocytes and was also found to a variable extent over the luteal bodies, varying between luteal masses rather than within individual bodies and parallelling the immunohistochemical findings (Pl. 2).

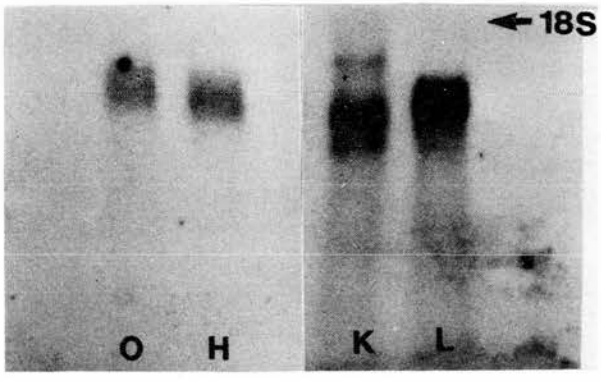
## DISCUSSION

Previous studies on ovarian 11β-HSD are few. Ghraf *et al.* (1975) reported no 11β-HSD bioactivity in the rat ovary but, in contrast, Murphy (1981*a*) found considerable bioactivity in human ovaries; both studies used similar methods.

We now report high 11β-HSD bioactivity in homogenized whole ovaries, in agreement with the findings of Murphy (1981*a*). The reason for the discrepancy between our data and those of Ghraf *et al.* (1975) is unclear but may relate to the very high pH (9·5) used in the latter study. The presence of 11β-HSD bioactivity is supported by the clear expression of mRNA encoding the 'liver-type' isoform of the enzyme. Using both immunohistochemistry and in-situ hybridization we have localized 11β-HSD mRNA antigen to oocytes and, to a lesser extent, to luteal bodies. The




TEXT-FIGURE 1. 11β-Hydroxysteroid dehydrogenase bioactivity (measured as fmol [$^3$H]11-dehydrocorticosterone formed from [$^3$H]corticosterone) in whole ovarian homogenates at protein concentrations of 0·0625 g/l (○; $n=3$), 0·25 g/l (□; $n=4$) and 0·5 g/l (▲; $n=3$). Values are means ± S.E.M.




TEXT-FIGURE 2. Autoradiograph of a Northern blot of total mRNA hybridized with $^{32}$P-labelled cDNA probes to 11β-hydroxysteroid dehydrogenase mRNA. O = ovary, H = hippocampus, K = kidney, L = liver. Note the presence of a single band in ovary corresponding to the one found in hippocampus and liver. Kidney shows multiple hybridizing mRNA species.

concordance of immunoreactivity and mRNA expression is in contrast to distal renal tubule (Edwards *et al.* 1988; Rundle, Funder, Lakshmi & Monder, 1989; Yau *et al.* 1991), where mRNA expression and bioactivity (Náray-Fejes-Tóth, Watlington & Fejes-Tóth, 1991) are found, but immunoreactivity (liver-type) is not detected. On the other hand, in hippocampus, cerebellum and vasculature, 11β-HSD mRNA expression colocalizes with immunoreactivity (Moisan *et al.* 1990*a*,*b*; Walker, Yau, Brett *et al.* 1991). It is thus likely that ovarian luteal bodies and oocytes express the liver-type mRNA and protein which may modulate glucocorticoid access to receptors rather than the distal convoluted tubule isoform which is completely protective of the colocalized mineralocorticoid receptor.

The interesting finding of high bioactivity localized to the oocyte itself, as indicated by in-situ hybridization and immunohistochemistry, implies modification of the oocyte glucocorticoid exposure and makes the presence of steroid receptors within the oocyte likely, as is the case in other 11β-HSD locations. Although glucocorticoids are believed to inhibit ovulation by inhibiting the preovulatory LH surge, our findings point to a possible local role of glucocorticoids in ovulation and/or oocyte differentiation. Interestingly, the specificity of 11β-HSD for 11β-hydroxyprogesterone and 11β-hydroxypregnenolone as well as their α-counterparts is much higher than for cortisol or corticosterone (Murphy, 1981*b*), raising the intriguing possibility that the substrate for ovarian 11β-HSD is not necessarily a glucocorticoid.

We were unable to find any indication of the presence of 11β-HSD in the granulosa cells, which

previously have been shown to contain glucocorticoid receptors (Schreiber *et al.* 1982) and to be metabolically regulated by glucocorticoids (Hsueh & Erickson, 1978; Adashi *et al.* 1981; Wang & Leung, 1989). This absence of 11β-HSD expression in the granulosa cells could be the result of aromatase activity generating oestrogen, which has been shown to inhibit potently activity of the liver-type 11β-HSD (Monder & Shackleton, 1984), as present in the ovary. Similarly, we did not demonstrate the presence of 11β-HSD in theca cells, although dexamethasone has been shown to alter their metabolism (reducing carbonyl reductase content) (Inazu *et al.* 1990). Our finding of 11β-HSD immunoreactivity and mRNA expression in the luteal cells but not the theca cells may be another example of cellular ontogeny, as we have shown in the skin (Teelucksingh *et al.* 1990), where 11β-HSD is not detected in the basal cell layer but is located in cells of the upper epidermis.

In summary, we have found 11β-HSD gene expression and bioactivity in the rat ovary, localized mainly in the oocyte and luteal masses. This suggests that glucocorticoids may exert a local modulatory role on ovarian function, which can be altered by cell-specific metabolism of steroids. The importance of 11β-HSD in ovarian function remains to be determined.

## ACKNOWLEDGEMENTS

We thank Dr C. Monder for generously providing the 11β-HSD antisera and cDNA clone. This work was supported by a Wellcome Trust/Royal Society of Edinburgh Senior Research Fellowship (J.R.S.) and a grant from the Scottish Hospital Endowments Research Trust.

## REFERENCES

Adashi, E. Y., Jones, P. B. C. & Hsueh, A. J. W. (1981). Synergistic effects of glucocorticoids on the stimulation of progesterone production by follicle-stimulating hormone in cultured rat granulosa cells. *Endocrinology* **109,** 1888–1894.

Agarwal, A. K., Monder, C., Eckstein, B. & White, P. C. (1989). Cloning and expression of rat cDNA encoding corticosteroid 11β-dehydrogenase. *Journal of Biological Chemistry* **264,** 18939–18943.

Albiston, A. L., Lock, P., Burger, H. G. & Krozowski, Z. S. (1990). Cloning and characterization of the rat α-inhibin gene. *Molecular and Cellular Endocrinology* **68,** 121–128.

Baldwin, D. M. & Sawyer, C. H. (1974). Effects of dexamethasone on LH release and ovulation in the cyclic rat. *Endocrinology* **94,** 1397–1403.

Beitins, I. Z., Bayard, F., Ances, I. G., Kowarski, A. & Migeon, C. J. (1973). The metabolic clearance rate, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy near term. *Pediatric Research* **7,** 509–519.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by guanidinum thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162,** 156–159.

Edwards, C. R. W., Stewart, P. M., Burt, D., Brett, L. P., McIntyre, M. A., Sutanto, W. S., de Kloet, E. R. & Monder, C. (1988). Localization of 11β-hydroxysteroid dehydrogenase – tissue-specific protector of the mineralocorticoid receptor. *Lancet* **ii,** 986–989.

Funder, J. W., Pearce, P. T., Smith, R. & Smith, A. L. (1988). Mineralocorticoid action: Target tissue specificity is enzyme, not receptor, mediated. *Science* **242,** 583–585.

Ghraf, R., Vetter, U., Zandveld, J. M. & Schriefers, H. (1975). Organ-specific ontogenesis of steroid hormone metabolizing enzyme activities in the rat. *Acta Endocrinologica* **79,** 192–201.

Hsueh, A. J. W. & Erickson, G. F. (1978). Glucocorticoid inhibition of FSH-induced estrogen production in cultured rat granulosa cells. *Steroids* **32,** 639–648.

Inazu, N., Iwata, N. & Satoh, T. (1990). Inhibitory effect of glucocorticoid and stimulatory effect of human chorionic gonadotropin on ovarian carbonyl reductase. *Life Sciences* **46,** 841–848.

Jia, X. C. & Hsueh, A. J. W. (1990). Synergistic effect of glucocorticoids and androgens on the hormonal induction of tissue plasminogen activator. Activity and messenger ribonucleic acid level in granulosa cells. *Molecular and Cellular Endocrinology* **68,** 143–151.

López Bernal, A., Anderson, A. B. M. & Turnbull, A. C. (1982). The lack of influence of labor on human placental 11β-hydroxysteroid dehydrogenase activity. *Journal of Clinical Endocrinology and Metabolism* **54,** 1251–1254.

Malbon, C. C. & Hadcock, J. R. (1988). Evidence that glucocorticoid response elements in the 5′-noncoding region of the hamster beta 2-adrenergic receptor gene are obligate for glucocorticoid regulation of receptor mRNA levels. *Biochemical and Biophysical Research Communications* **154,** 676–681.

Moisan, M. P., Seckl, J. R., Brett, L. P., Monder, C., Agarwal, A. K., White, P. C. & Edwards, C. R. W. (1990*a*). 11Beta-hydroxysteroid dehydrogenase messenger ribonucleic acid expression, bioactivity and immunoreactivity in rat cerebellum. *Journal of Neuroendocrinology* **2,** 853–858.

Moisan, M. P., Seckl, J. R. & Edwards, C. R. W. (1990*b*). 11β-Hydroxysteroid dehydrogenase bioactivity and messenger RNA expression in rat forebrain: Localization in hypothalamus, hippocampus, and cortex. *Endocrinology* **127,** 1450–1455.

Monder, C. & Lakshmi, M. (1990). Corticosteroid 11β-dehydrogenase of rat tissues: Immunological studies. *Endocrinology* **126,** 2435–2443.

Monder, C. & Shackleton, C. H. L. (1984). 11β-Hydroxysteroid dehydrogenase; fact or fancy? *Steroids* **44,** 383–417.

Murphy, B. E. P. (1981*a*). Ontogeny of cortisol–cortisone interconversion in human tissues: A role for cortisone in human fetal development. *Journal of Steroid Biochemistry* **14,** 811–817.

Murphy, B. E. P. (1981*b*). Specificity of human 11β-hydroxysteroid dehydrogenase. *Journal of Steroid Biochemistry* **14,** 807–809.

Náray-Fejes-Tóth, A., Watlington, C. O. & Fejes-Tóth, G. (1991). 11β-Hydroxysteroid dehydrogenase activity in the renal target cells of aldosterone. *Endocrinology* **129,** 17–21.

Phillips, D. M., Lakshmi, V. & Monder, C. (1989). Corticosteroid 11β-dehydrogenase in rat testis. *Endocrinology* **125,** 209–216.

Reich, R., Miskin, R. & Tsafriri, A. (1985). Follicular plasminogen activator: Involvement in ovulation. *Endocrinology* **116,** 516–521.

Rundle, S. E., Funder, J. W., Lakshmi, V. & Monder, C. (1989). The intrarenal localization of mineralocorticoid receptors and 11β-dehydrogenase: immunocytochemical studies. *Endocrinology* **125,** 1700–1704.

Schreiber, J. R., Nakamura, K. & Erickson, G. F. (1982). Rat ovary glucocorticoid receptor: Identification and characterization. *Steroids* **39,** 569–584.

Sternberger, L. A., Hardy, P. H. Jr., Cuculis, J. J. & Meyer, H. G. (1970). The unlabeled antibody–enzyme method of immunohistochemistry. Preparation and properties of soluble antigen–antibody complex (horseradish peroxidase–antihorseradish peroxidase) and its use in identification of spirochetes. *Journal of Histochemistry and Cytochemistry* **18,** 315–333.

Tannin, G. M., Agarwal, A. K., Monder, C., New, M. I. & White, P. C. (1991). The human gene for 11β-hydroxysteroid dehydrogenase. Structure, tissue distribution, and chromosomal localization. *Journal of Biological Chemistry* **266,** 16653–16658.

Teelucksingh, S., Mackie, A. D. R., Burt, D., McIntyre, M. A., Brett, L. P. & Edwards, C. R. W. (1990). Potentiation of hydrocortisone activity in skin by glycyrrhetinic acid. *Lancet* **335,** 1060–1063.

Walker, B. R., Yau, J. L., Brett, L. P., Seckl, J. R., Monder, C., Williams, B. C. & Edwards, C. R. W. (1991). 11β-Hydroxysteroid dehydrogenase in vascular smooth muscle and heart: Implications for cardiovascular responses to glucocorticoids. *Endocrinology* **129,** 3305–3312.

Wang, C. & Leung, A. (1989). Glucocorticoids stimulate plasminogen activator production by rat granulosa cells. *Endocrinology* **124,** 1595–1601.

Yau, J. L. W., Van Haarst, A. D., Moisan, M. P., Fleming, S. Edwards, C. R. W. & Seckl, J. R. (1991). 11β-hydroxysteroid dehydrogenase mRNA expression in rat kidney. *American Journal of Physiology* **29,** F764–F767.

## DESCRIPTION OF PLATES

### Plate 1

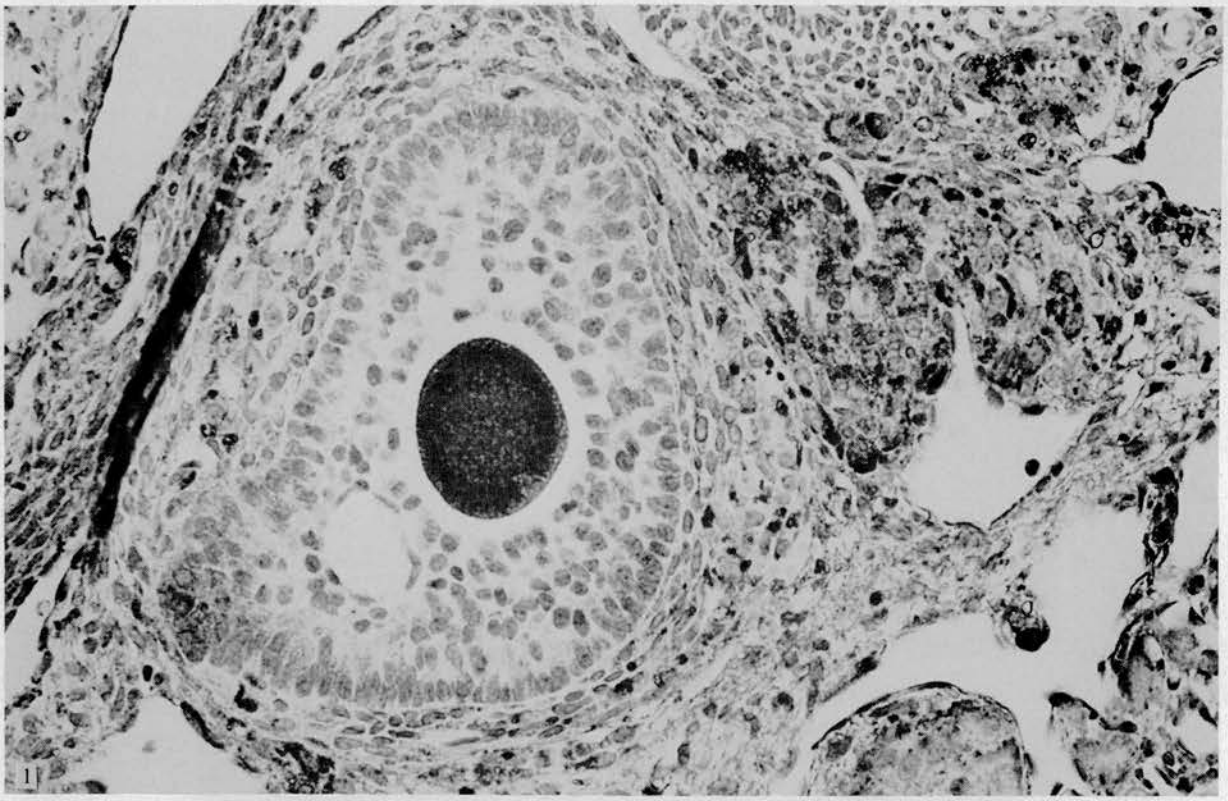
FIGURE 1. Immunostaining of rat ovary using antiserum to purified rat liver 11β-hydroxysteroid dehydrogenase, displaying marked staining of the oocyte (×400).

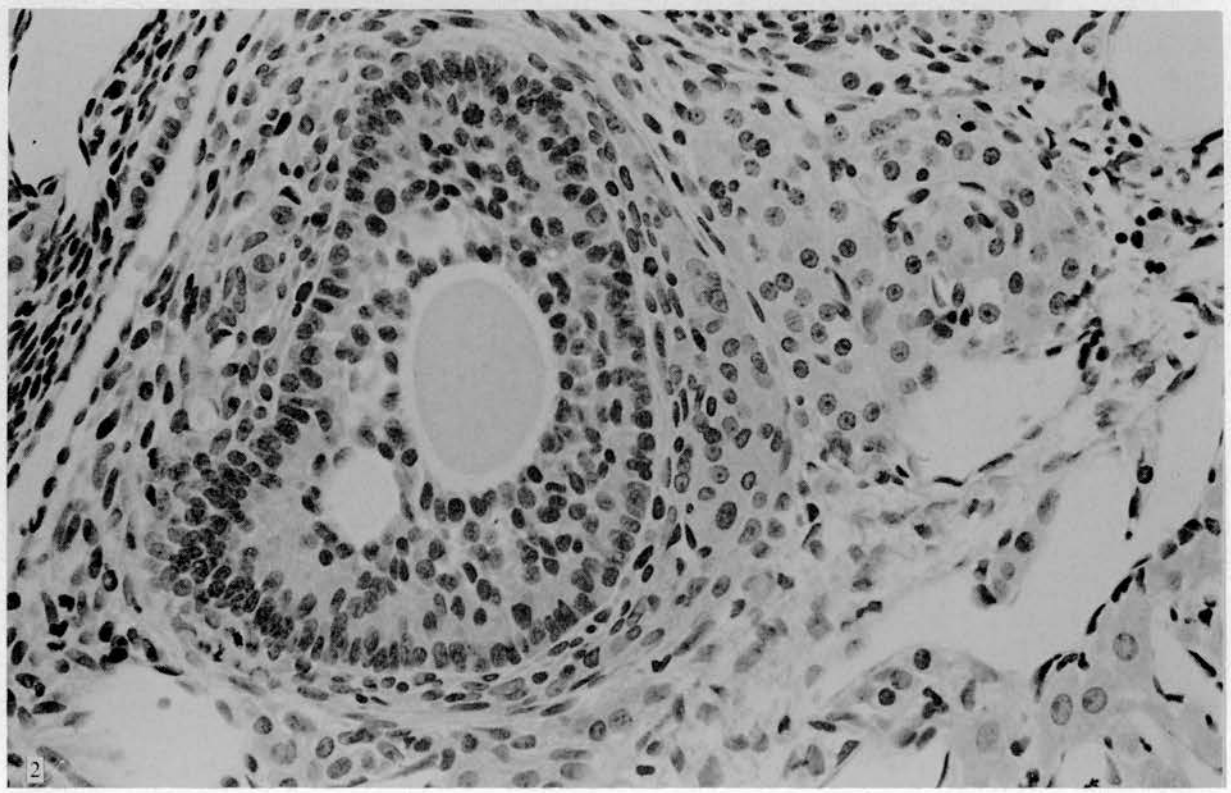
FIGURE 2. Control using preimmune serum, showing no immunostaining (×400).

### Plate 2

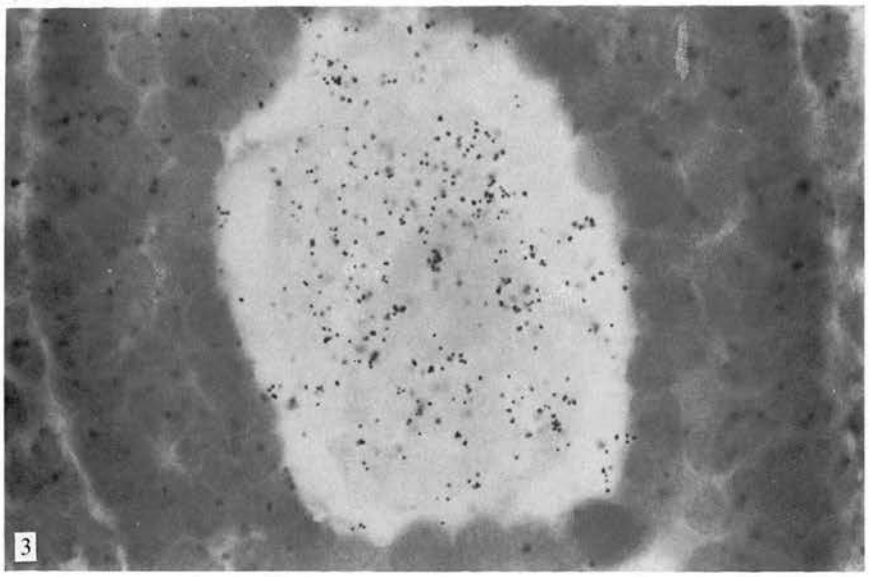
FIGURE 3. In-situ hybridization showing a concentration of silver grains over the oocyte indicating hybridization of the cRNA probe containing $^{35}$S-labelled UTP to 11β-hydroxysteroid dehydrogenase mRNA (×600).

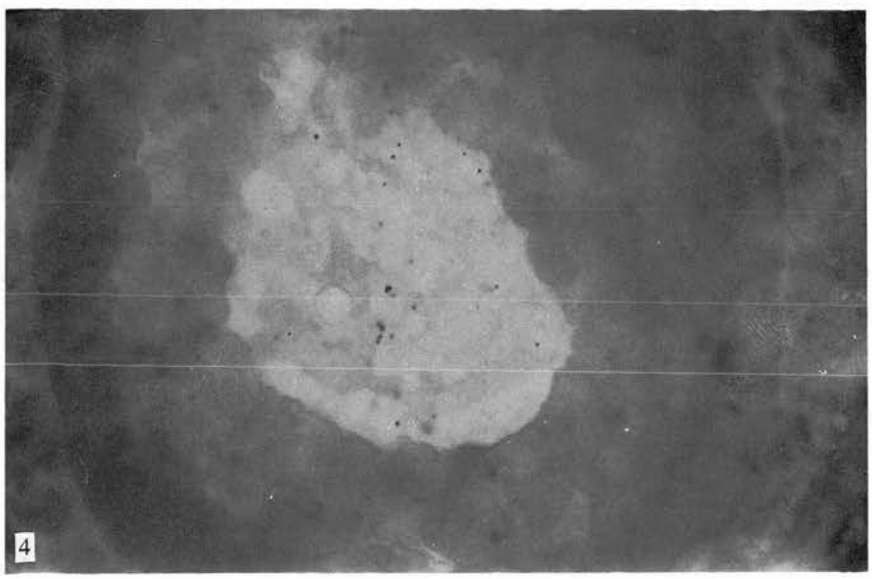
FIGURE 4. In-situ hybridization using 'sense' probe, showing no silver grain concentration (×600).




(Facing p. 58)


3


4

# The 11β-hydroxysteroid dehydrogenase inhibitor glycyrrhetinic acid affects corticosteroid feedback regulation of hypothalamic corticotrophin-releasing peptides in rats

**J. R. Seckl, R. C. Dow*, S. C. Low, C. R. W. Edwards** and **G. Fink***

Department of Medicine, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU, U.K.
*MRC Brain Metabolism Unit, 1 George Square, Edinburgh EH8 9JZ, U.K.

RECEIVED 16 July 1992

## ABSTRACT

Steroid-metabolizing enzymes modulate the effects of androgens on brain differentiation and function, but no similar enzymatic system has been demonstrated for adrenocorticosteroids which exert feedback control on the hypothalamus. 11β-Hydroxysteroid dehydrogenase (11β-OHSD) rapidly metabolizes physiological glucocorticoids (corticosterone, cortisol) to inactive products, thereby regulating glucocorticoid access to peripheral mineralocorticoid and glucocorticoid receptors in a site-specific manner. Using in-situ hybridization, we found expression of 11β-OHSD mRNA in neurones of the hypothalamic paraventricular nucleus (PVN) where corticotrophin-releasing factor-41 (CRF-41) is synthesized and from where it is released into hypophysial portal blood. Administration of glycyrrhetinic acid (GE), a potent 11β-OHSD inhibitor, decreased CRF-41 release into hypophysial portal blood in the presence of unchanged circulating glucocorticoid levels, suggesting that 11β-OHSD regulates the effective corticosterone feedback signal to CRF-41 neurones. These effects of GE were not observed in adrenalectomized animals, demonstrating dependence on adrenal products. In contrast, GE led to two- to threefold increases in arginine vasopressin and oxytocin release into portal blood, effects also dependent upon intact adrenal glands. These results suggest that 11β-OHSD in the PVN, and possibly other sites, may represent a novel and important control point of corticosteroid feedback on CRF-41 release *in vivo*.
*Journal of Endocrinology* (1993) **136,** 471–477

## INTRODUCTION

The neural control of pituitary corticotrophin (ACTH) secretion is mediated by corticotrophin-releasing factor-41 (CRF-41) and arginine vasopressin (AVP), and under certain circumstances oxytocin, synthesized in the hypothalamic paraventricular nucleus (PVN) (Antoni, 1986; Rivier & Plotsky, 1986; Fink *et al.* 1988; Tannahill *et al.* 1991). Corticosteroids, released in response to ACTH, complete the regulatory loop by a negative feedback action at the brain–pituitary level. However, the mechanism of corticosteroid negative feedback remains imperfectly understood with differences in the effects of physiological and synthetic glucocorticoids and the conditions (acute or chronic) of the experiment (Plotsky & Vale, 1984; Plotsky *et al.* 1986; Rivier & Plotsky, 1986; Plotsky & Sawchenko, 1987; Fink *et al.* 1988; Dallman *et al.* 1989; Sheward & Fink, 1991; Tannahill *et al.* 1991). Corticosteroid actions are mediated by intracellular glucocorticoid receptors (GR, type II) and mineralocorticoid receptors (MR, type I) (McEwen *et al.* 1986*a*). GR are highly expressed in anterior pituitary corticotrophs and in the PVN, where they co-localize with CRF-41-immunoreactive cells (Fuxe *et al.* 1985). MR-like binding sites are also found in pituitary and hypothalamus and MR mRNA expression has been detected in the PVN (McEwen *et al.* 1986*b*; Swanson & Simmons, 1989).

GR and MR show a high degree of structural homology. Indeed, *in vitro*, MR bind the mineralocorticoid aldosterone and the physiological glucocorticoids cortisol and corticosterone with equal affinity (Arriza *et al.* 1988). *In vivo*, aldosterone selectivity of

  *Printed in Great Britain*
0022–0795/93/0136–0471 $02.00/0

MR in the kidney in the face of a 1000-fold excess of circulating glucocorticoid is secured by 11β-hydroxysteroid dehydrogenase (11β-OHSD) which rapidly converts corticosterone or cortisol into inactive 11-keto products (11-dehydrocorticosterone or cortisone respectively), thereby preventing these physiological glucocorticoids from gaining access to peripheral GR and MR (Edwards *et al.* 1988; Funder *et al.* 1988; Teelucksingh *et al.* 1990; Yau *et al.* 1991). 11β-OHSD bioactivity and mRNA expression are also present in various subregions of the brain, including the hypothalamus (Moisan *et al.* 1990*b*) where some MR are selective for aldosterone *in vivo* (McEwen *et al.* 1986*a*). The aim of the present study was to determine (i) whether 11β-OHSD is expressed in the PVN and (ii) if so, whether it plays a role in the negative feedback regulation by corticosterone of hypothalamic CRF-41 secretion into hypophysial portal plasma.

## MATERIALS AND METHODS

### In-situ hybridization histochemistry

To determine whether mRNAs encoding 11β-OHSD and MR are expressed in PVN cells, in-situ hybridization was performed using non-isotopic cRNA probes as previously described (Yau *et al.* 1991). Briefly, fresh frozen coronal brain sections (10 μm) were post-fixed in 4% paraformaldehyde/phosphate buffer (0·1 mol/l) and washed twice in 2 × standard sodium citrate (SSC) prior to hybridization. T3 polymerase (Gibco BRL, Paisley, Strathclyde, U.K.) was used to transcribe 598 bp antisense cRNA probes from StyI-linearized pBluescript vector containing 11β-OHSD cDNA (Agarwal *et al.* 1989). For MR mRNA probes, a 513 bp EcoR1 fragment of rat cDNA in pGEM4 (Arriza *et al.* 1988) was linearized with HindIII and transcribed with SP6 polymerase. Biotin-11-UTP (1 mmol/l) was added to the in-vitro transcription reaction. Hybridization and high stringency washes were performed as described (Yau *et al.* 1991). Biotin was detected by exposure to avidin–biotin peroxidase complex (ABC; Vector Laboratories, Peterborough, Cambs, U.K.) for 30 min. Sensitivity was increased by subsequent incubation with biotinylated anti-avidin (1:250) for 60 min and repeat exposure to ABC. 3,3′-Diaminobenzidine was added as chromogen. Cell nuclei were counterstained with methyl green. Controls sections were (i) hybridized with identically labelled 'sense' RNA probes and (ii) incubated in buffer alone (i.e. without cRNA probes) to determine the endogenous biotin signal. Positive controls were provided by determining, in the same section, hybridization of the cRNA probes to the overlying dorsal hippocampus, which expresses both 11β-OHSD and MR mRNAs highly (Moisan *et al.* 1990*b*; Seckl *et al.* 1990).

### Hypophysial portal blood sampling

Male Wistar rats (200 g body weight) were maintained under conditions of controlled temperature (22 °C) and lighting (lights on 05.00–19.00 h) and allowed free access to diet and tap water. Animals were anaesthetized with sodium pentobarbitone (36–40 mg/kg body weight, i.p.) and the hypophysial portal vessels exposed by a transpharyngeal approach as previously described (Fink *et al.* 1988; Sheward & Fink, 1991; Tannahill *et al.* 1991). Rats were then injected subcutaneously with glycyrrhetinic acid (GE; Aldrich Chemical Company, Gillingham, Dorset, U.K.; 5 mg in ethanol, $n=10$) or ethanol vehicle ($n=15$) at 0 and 45 min. Glycyrrhetinic acid, the active component of liquorice, inhibits central 11β-OHSD potently (Moisan *et al.* 1990*a*). Portal blood was collected, from 0 min, for two consecutive 45-min periods into ice-cold tubes containing aprotinin (10 μl/100 μl blood).

In order to determine whether the effects of GE were dependent on the presence of endogenous corticosteroids which are substrates for 11β-OHSD, a second study was carried out in which rats were adrenalectomized under halothane anaesthesia and then injected daily with 0·9 % (w/v) NaCl ($n=16$) or dexamethasone (Sigma, Poole, Dorset, U.K.; 0·2 mg/kg, s.c., $n=6$) for 10 days. Adrenalectomized animals were given saline (containing 2% dextrose) instead of tap water to drink. Rats were then anaesthetized with sodium pentobarbitone and injected with GE as above (eight adrenalectomized rats and all dexamethasone-treated animals) or ethanol vehicle (the remaining eight adrenalectomized rats) immediately before each hypophysial portal blood sampling period.

Portal blood samples were centrifuged and plasma was stored at −40 °C before peptide assay. CRF-41, AVP and oxytocin were estimated in portal plasma using specific and sensitive radioimmunoassays (Fink *et al.* 1988). Peripheral blood samples were obtained at the beginning of the experiment for estimation of plasma ACTH (to ensure successful adrenalectomy) and at the end of the study for measurement of plasma corticosterone, osmolality and glucose. ACTH and corticosterone were estimated by specific radioimmunoassays (Fink *et al.* 1988), plasma osmolality and glucose were measured using the freezing-point depression and glucose oxidase/peroxidase methods respectively. Data were compared by analysis of variance or unpaired Student's *t*-tests, as appropriate. Significance was set at $P<0·05$. Values are means ± S.E.M.

## RESULTS

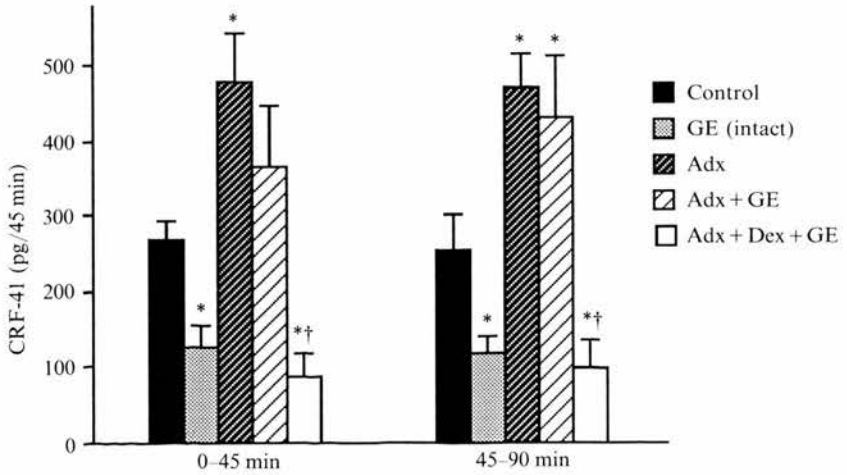
In-situ hybridization histochemistry showed 11β-OHSD mRNA expression in 70 ± 5% of magnocellular PVN neurones (out of a total of 267 magnocellular cells counted in five separate sections) and 72 ± 8% of 153 parvicellular PVN neurones (Plate). MR mRNA was expressed in 68 ± 7% of 151 magnocellular and 66 ± 7% of 262 parvicellular PVN neurones (Plate). There was high expression of MR and 11β-OHSD mRNAs in the dorsal hippocampus (expression was found in the vast majority of dentate gyrus granule cells and pyramidal cells of the cornu ammonis; data not shown). Sense RNA probes did not hybridize to cells in the PVN (Plate) or hippocampus (data not shown) and there was little or no 'endogenous biotin' signal in control sections (Plate).

Administration of GE significantly reduced the amount of CRF-41 released into portal blood by more than 50% (Text-fig. 1), although peripheral plasma corticosterone levels remained unchanged (Table 1). In contrast, GE treatment was associated with a 2·5- to 3·5-fold increase in portal plasma AVP and oxytocin levels (Text-fig. 2) which could not be explained by changes in plasma osmolality or glucose (Table 1).

To determine whether the GE-mediated changes in portal peptide content were due to 11β-OHSD inhibition and thus dependent on the presence of circulating substrate (corticosterone), GE was administered to chronically (10 days) adrenalectomized rats, some of which were also treated with the synthetic glucocorticoid dexamethasone which is not metabolized by 11β-OHSD. Adrenalectomized controls received vehicle instead of GE. Adrenalectomy alone led to elevated CRF and AVP, but not oxytocin content in portal blood when compared with intact controls (Text-figs 1 and 2). Adrenalectomy prevented the fall in portal CRF-41 and the rise in AVP and oxytocin caused by GE (Text-figs 1 and 2) and GE administration did not exert any effect significantly different from adrenalectomy alone, demonstrating that the effect of GE was dependent on the normal secretion of endogenous adrenal products and suggesting that the effect of GE is not a direct pharmacological one or simply stress-related. Further evidence that the lack of response to GE in adrenalectomized rats was due to the absence of endogenous glucocorticoids was provided by the fact that the hypothalamic-pituitary-adrenal axis remained responsive to glucocorticoid negative feedback, as shown by the significant reduction of CRF-41, AVP and oxytocin release into portal blood (Text-fig. 1) in adrenalectomized animals treated with GE plus dexamethasone when compared with adrenalectomy alone (or adrenalectomy plus GE).

## DISCUSSION

These results show that 11β-OHSD mRNA is expressed in a large proportion of the magnocellular




TEXT-FIGURE. 1. Effects of glycyrrhetinic acid (GE; 5 mg s.c. at 0 and 45 min, $n = 10$) or vehicle ($n = 15$) on the output of corticotrophin-releasing factor-41 (CRF-41) into hypophysial portal blood collected over two consecutive 45-min periods in rats anaesthetized with sodium pentobarbitone. Further rats were adrenalectomized (Adx) and given dexamethasone (Dex; 0·2 mg/kg per day) replacement or vehicle for 10 days prior to portal blood sampling ($n = 6$–8 per group). Values are means ± S.E.M. *$P < 0·05$ compared with control; †$P < 0·05$ compared with adrenalectomized animals.

TABLE 1. Effects of glycyrrhetinic acid (GE; 5 mg; administered s.c. at 0 and 45 min) or vehicle on peripheral plasma corticosterone, osmolality and glucose concentrations after 90 min in anaesthetized rats. Control and GE-treated rats were adrenally intact. Further animals were adrenalectomized (Adx) and were given vehicle, GE or dexamethasone replacement (Dex) and GE. Values are means ± S.E.M. ($n = 6$–8 per group)

| | Control | GE | Adx | Adx + GE | Adx + Dex + GE |
|---|---|---|---|---|---|
| Corticosterone (μg/l) | 344 ± 16 | 274 ± 64 | < DL | < DL | < DL |
| Osmolality (mosm/kg) | 295 ± 2 | 295 ± 3 | | | |
| Glucose (mmol/l) | 10 ± 0·7 | 9·7 ± 1·9 | | | |

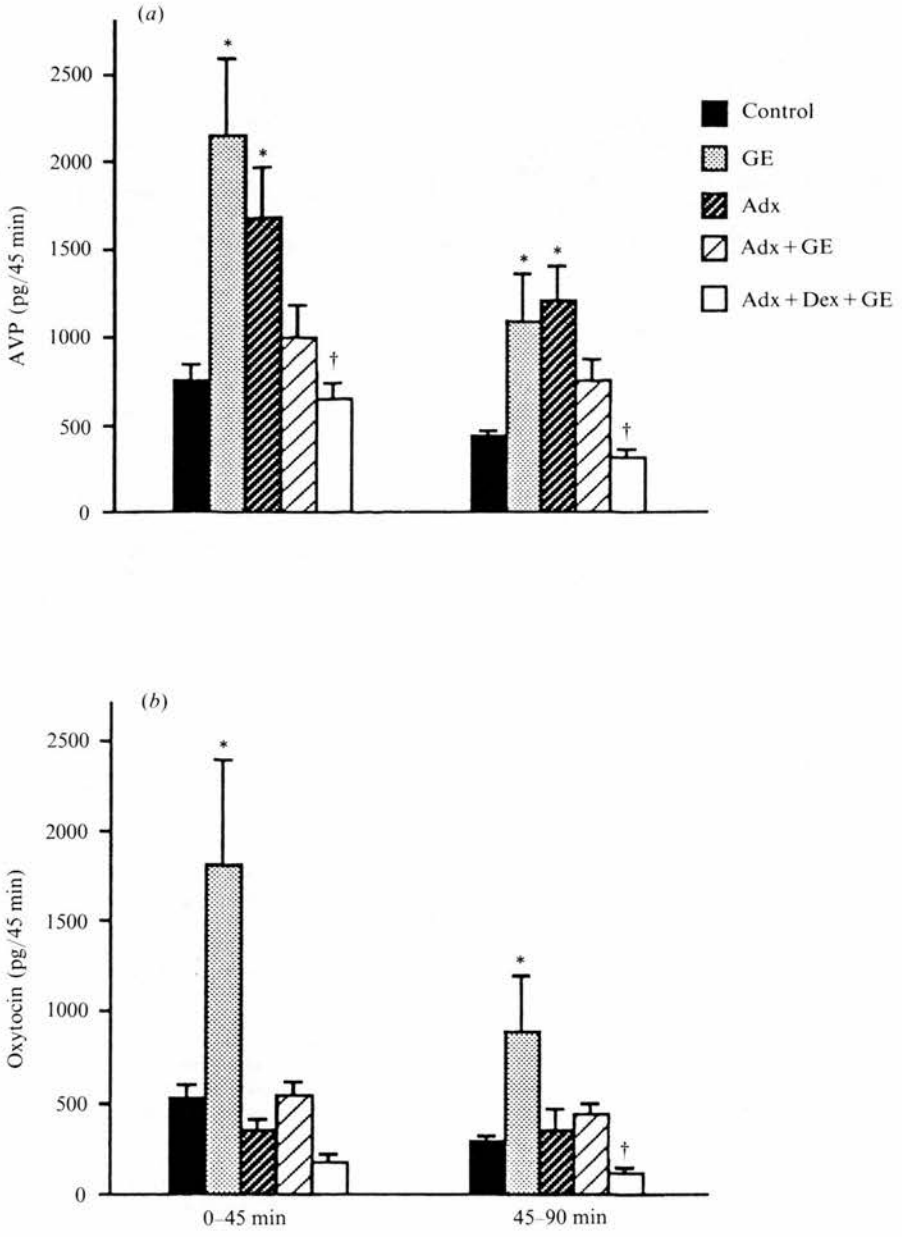
< DL = below assay detection limit.

and parvicellular neurones of the PVN and suggest that the activity of 11β-OHSD affects glucocorticoid negative feedback. This assertion is based on (i) our in-situ hybridization data and (ii) the fact that inhibition of 11β-OHSD by GE in intact rats reduced CRF-41 release into portal blood. The latter occurred in the absence of any significant change in plasma corticosterone concentrations and GE had no significant effect on CRF-41 release in adrenalectomized rats. This suggests that the reduced CRF-41 release in intact rats treated with GE was due to the negative feedback of corticosterone which accumulated as a consequence of decreased metabolism to its inactive 11-dehydro products. Although it is possible that the increased hypothalamic drive brought about by adrenalectomy is sufficient to overcome a direct effect (i.e. not via corticosteroid metabolism) of GE seen in intact rats, the effect of GE on CRF-41 release is similar in magnitude to that seen with chronic high-dose dexamethasone treatment of adrenalectomized rats, suggesting that 11β-OHSD provides a potent and important degree of regulation of corticosteroid feedback on CRF-41.

Dexamethasone is a more potent GR-specific ligand than corticosterone but has little effect on MR and since dexamethasone does not appear to exert any short-term regulatory influence on portal CRF-41 release (Fink *et al.* 1988), it is probable that the effect of corticosterone on unstimulated CRF-41 release is mediated via MR. This accords with previous studies of the effects of corticosteroid in the brain where dexamethasone is less effective than corticosterone in inhibiting ACTH secretion (Levin *et al.* 1988; Dallman *et al.* 1989). Physiological corticosterone feedback on the hypothalamic-pituitary-adrenal axis, during both the diurnal peak and trough, is mediated, at least in part, by MR (Dallman *et al.* 1989; Ratka *et al.* 1989), aldosterone is selectively concentrated in the hypothalamus (Yongue & Roy, 1987) and excess corticosterone cannot displace hypothalamic aldosterone binding in adrenalectomized rats (McEwen *et al.* 1986*b*), providing further evidence to suggest that 11β-OHSD protects hypothalamic MR from corticosterone *in vivo*.

Although GE potently inhibits both peripheral and central 11β-OHSD (Edwards *et al.* 1988; Moisan *et al.* 1990*a*), 11β-OHSD is fairly widely distributed in the brain and since GE is a highly lipophilic steroid-like substance, and is therefore also likely to be widely distributed in the brain, the present studies do not pin-point precisely where the enzyme exerts its effect on the corticosteroid control of CRF-41 release. However, as (i) the PVN produces about 90% of the CRF-41 released into hypophysial portal blood (Antoni *et al.* 1990), (ii) stimulation of the PVN, perhaps rather than other brain areas, results in CRF-41 release into portal blood (Tannahill *et al.* 1991) and (iii) the PVN contains 11β-OHSD and GR (Fuxe *et al.* 1985) as well as MR (Swanson & Simmons, 1989 and Plate) it is likely that the PVN represents a major, although not necessarily the only, site at which 11β-OHSD exerts a controlling influence on the corticosteroid negative feedback system.

The stimulation of AVP and oxytocin release into portal blood by GE is puzzling, but as in the case of the GE-induced decrease in CRF-41 release, the effect was not seen in adrenalectomized rats and, therefore, would appear to depend on an adrenal product; indeed GE tended to decrease portal plasma AVP content in adrenalectomized animals. In addition to inhibiting 11β-OHSD, GE also inhibits 5β-reductase and 3β-hydroxysteroid dehydrogenase (Latif *et al.* 1990) which would result in the increased accumulation of aldosterone, 5α-dihydroxyaldosterone and possibly other steroids in the brain. Although these products bind to MR (Latif *et al.* 1990), and might therefore be expected merely to amplify any effect of corticosterone on 11β-OHSD-protected MR, they might also conceivably stimulate rather than inhibit AVP and oxytocin release, perhaps via MR at other sites or by GR antagonism. Dissociation between CRF-41, AVP and oxytocin release into portal blood is not unique, in that clear-cut dissociation between the release of these three neuropeptides into portal blood has been found to occur under several conditions (Rivier & Plotsky, 1986; Plotsky & Sawchenko, 1987; Fink *et al.* 1988; Antoni *et al.* 1990; Sheward *et al.* 1990; Tannahill *et al.* 1991). Although neurones




TEXT-FIGURE 2. Effects of glycyrrhetinic acid (GE; 5 mg s.c. at 0 and 45 min, $n=10$) or vehicle ($n=15$) on the output of (*a*) AVP and (*b*) oxytocin into hypophysial portal blood collected over two consecutive 45-min periods in rats anaesthetized with sodium pentobarbitone. Further rats were adrenalectomized (Adx) and given dexamethasone (Dex; 0·2 mg/kg per day) replacement or vehicle for 10 days prior to portal blood sampling ($n=6$–8 per group). Values are means ± S.E.M. $^{*}P<0{\cdot}05$ compared with control; $^{\dagger}P<0{\cdot}05$ compared with adrenalectomized animals.

from both parvicellular PVN (AVP- and CRF-containing cells projecting to the median eminence) and magnocellular PVN and supraoptic nucleus (AVP and oxytocin cells projecting to the neurohypophysis) contribute to AVP in portal plasma (Antoni *et al.* 1990), there is no evidence that GE affects plasma osmolality or glucose (present data) or blood pressure (Seckl *et al.* 1991), excluding conventional magnocellular AVP activators (osmotic challenge, hypoglycaemia, hypotension) which might explain increased AVP and oxytocin release. Thus, the GE-stimulated release of AVP and oxytocin is an intriguing and potentially important finding which requires further investigation.

In summary, the present data imply that 11β-OHSD and possibly other corticosteroid-metabolizing enzymes in the brain play a major role in the negative feedback control by corticosteroids of CRF-41 release into hypophysial portal blood. The unexpected finding that GE can stimulate AVP and oxytocin release in adrenal intact, but not adrenalectomized, rats is another example of the dissociation between the release of CRF-41 and the neurohypophysial peptides.

## ACKNOWLEDGEMENTS

We thank Dr C. Monder, Dr R. Evans and Dr J. Arriza for generously providing the cDNA clones used, H. Dick, J. Bennie and S. Carroll for carrying out the radioimmunoassays and the Scottish Antibody Production Unit, Carluke, Scotland for radioimmunoassay materials. This work was supported by a Wellcome Trust/Royal Society of Edinburgh Senior Research Fellowship (J.R.S.) and grants (to J.R.S.) from the Scottish Hospital Endowments Research Trust and the Sir Stanley and Lady Davidson Research Fund.

## REFERENCES

Agarwal, A. K., Monder, C., Eckstein, B. & White, P. C. (1989). Cloning and expression of rat cDNA encoding corticosteroid 11dehydrogenase. *Journal of Biological Chemistry* **264,** 18939–18943.

Antoni, F. A. (1986). Hypothalamic control of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor. *Endocrine Reviews* **7,** 351–378.

Antoni, F. A., Fink, G. & Sheward, W. J. (1990). Corticotrophin-releasing peptides in rat hypophysial portal blood after paraventricular lesions: a marked reduction in the concentration of corticotrophin-releasing factor-41, but no change in vasopressin. *Journal of Endocrinology* **125,** 175–183.

Arriza, J. L., Simerly, R. B., Swanson, L. W. & Evans, R. M. (1988). The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. *Neuron* **1,** 887–900.

Dallman, M. F., Levin, N., Cascio, C. S., Akana, S. F., Jacobson, L. & Kuhn, R. W. (1989). Pharmacological evidence that the inhibition of diurnal adrenocorticotropin secretion by corticosteroids is mediated via type I corticosterone-preferring receptors. *Endocrinology* **124,** 2844–2850.

Edwards, C. R. W., Stewart, P. M., Burt, D., Brett, L., McIntyre, M. A., Sutanto, W. S., de Kloet, E. R. & Monder, C. (1988). Localisation of 11β-hydroxysteroid dehydrogenase-tissue specific protector of the mineralocorticoid receptor. *Lancet* **ii,** 986–989.

Fink, G., Robinson, I. C. A. F. & Tannahill, L. A. (1988). Effects of adrenalectomy and glucocorticoids on the peptides CRF-41, AVP and oxytocin in rat hypophysial portal blood. *Journal of Physiology* **401,** 329–345.

Funder, J. W., Pearce, P. T., Smith, R. & Smith, A. I. (1988). Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science* **242,** 583–585.

Fuxe, K., Wikstrom, A.-C., Okret, S., Agnati, L. F., Harfstrand, A., Yu, Z.-Y., Granholm, L., Zoli, M., Vale, W. & Gustafsson, J.-A. (1985). Mapping of glucocorticoid receptor immunoreactive oxytocin in the rat tel- and diencephalon using a monoclonal antibody against rat liver glucocorticoid receptor. *Endocrinology* **117,** 1803–1812.

Latif, S., Conca, T. & Morris, D. (1990). The effects of the liquorice derivative, glycyrrhetinic acid on hepatic 3α- and 3β-hydroxysteroid dehydrogenases and 5α- and 5β-reductase pathways of metabolism of aldosterone in male rats. *Steroids* **55,** 52–58.

Levin, N., Shinsako, J. & Dallman, M. F. (1988). Corticosterone acts on the brain to inhibit adrenalectomy-induced adrenocorticotropin secretion. *Endocrinology* **122,** 694–701.

McEwen, B. S., de Kloet, E. R. & Rostene, W. (1986*a*). Adrenal steroid receptors and action in the nervous system. *Physiological Reviews* **66,** 1121–1188.

McEwen, B. S., Lambdin, L. T., Rainbow, T. C. & De Nicola, A. F. (1986*b*). Aldosterone effects on salt appetite in adrenalectomised rats. *Neuroendocrinology* **43,** 38–43.

Moisan, M.-P., Seckl, J. R., Brett, L. P., Monder, C., Agarwal, A. K., White, P. C. & Edwards, C. R. W. (1990*a*). 11β-Hydroxysteroid dehydrogenase messenger ribonucleic acid expression, bioactivity and immunoreactivity in rat cerebellum. *Journal of Neuroendocrinology* **2,** 853–858.

Moisan, M.-P., Seckl, J. R. & Edwards, C. R. W. (1990*b*). 11β-Hydroxysteroid dehydrogenase bioactivity and messenger RNA expression in rat forebrain: localization in hypothalamus, hippocampus and cortex. *Endocrinology* **127,** 1450–1455.

Plotsky, P. M., Otto, S. & Sapolsky, R. M. (1986). Inhibition of immunoreactive corticotropin-releasing factor secretion into the hypophysial portal circulation by delayed glucocorticoid feedback. *Endocrinology* **119,** 1126–1130.

Plotsky, P. M. & Sawchenko, P. E. (1987). Hypophysial-portal plasma levels, median eminence content and immunostaining of corticotropin-releasing factor, arginine vasopressin and oxytocin after pharmacological adrenalectomy. *Endocrinology* **120,** 1361–1369.

Plotsky, P. M. & Vale, W. (1984). Hemorrhage-induced secretion of corticotropin-releasing factor-like immunoreactivity into the rat hypophysial portal circulation and its inhibition by glucocorticoids. *Endocrinology* **114,** 164–169.

Ratka, A., Sutanto, W., Bloemers, M. & de Kloet, E. R. (1989). On the role of brain mineralocorticoid (type I) and glucocorticoid (type II) receptors in neuroendocrine regulation. *Neuroendocrinology* **50,** 117–123.

Rivier, C. L. & Plotsky, P. M. (1986). Mediation by corticotropin-releasing factor (CRF) of adenohypophysial hormone secretion. *Annual Review of Physiology* **48,** 475–496.

Seckl, J. R., Dickson, K. L. & Fink, G. (1990). 5,7-Dihydroxytryptamine decreases hippocampal glucocorticoid and mineralocorticoid receptor mRNA expression. *Journal of Neuroendocrinology* **2,** 911–916.

Seckl, J. R., Kelly, P. A. T. & Sharkey, J. (1991). Glycyrrhetinic acid, an inhibitor of 11β-hydroxysteroid dehydrogenase, alters local cerebral glucose utilization *in vivo*. *Journal of Steroid Biochemistry and Molecular Biology* **39,** 777–779.

Sheward, W. J., Coombes, J. E., Bicknell, R. J., Fink, G. & Russell, J. A. (1990). Release of oxytocin but not corticotrophin-releasing factor-41 into rat hypophysial portal vessel blood can be made opiate dependent. *Journal of Endocrinology* **124,** 141–150.

Sheward, W. J. & Fink, G. (1991). Effects of corticosterone on the secretion of corticotrophin-releasing factor, arginine vasopressin and oxytocin into hypophysial portal blood in long-term hypophysectomized rats. *Journal of Endocrinology* **129,** 91–98.

Swanson, L. W. & Simmons, D. M. (1989). Differential steroid hormone and neural influences on peptide mRNA levels in CRH cells of the paraventricular nucleus: a hybridization histochemical study in the rat. *Journal of Comparative Neurobiology* **285,** 413–435.

Tannahill, L. A., Sheward, W. J., Robinson, I. C. A. F. & Fink, G. (1991). Corticotrophin-releasing factor-41, vasopressin and oxytocin release into hypophysial portal blood in the rat: effects of electrical stimulation of the hypothalamus, amygdala and hippocampus. *Journal of Endocrinology* **129,** 99–107.

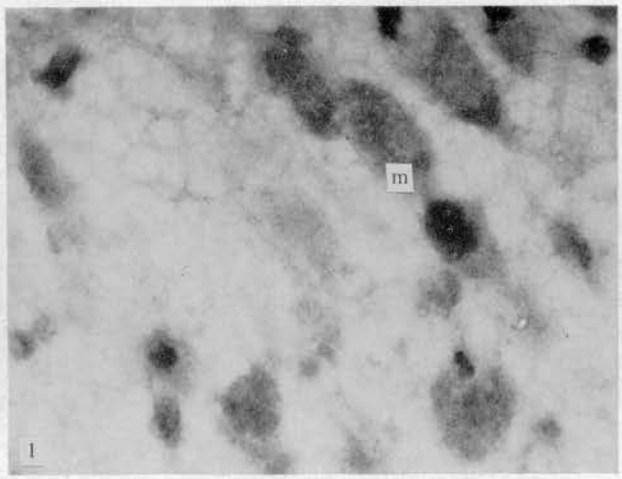
Teelucksingh, S., Mackie, A., Burt, D., McIntyre, M., Brett, L. & Edwards, C. (1990). Potentiation of hydrocortisone activity in skin by glycyrrhetinic acid. *Lancet* **335,** 1060–1063.

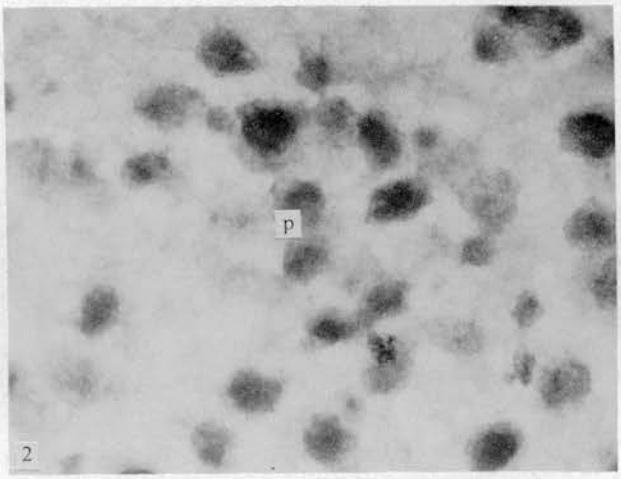
Yau, J. L. W., van Haarst, A. D., Moisan, M.-P., Fleming, S., Edwards, C. R. W. & Seckl, J. R. (1991). 11β-Hydroxysteroid dehydrogenase messenger RNA expression in rat kidney. *American Journal of Physiology* **260,** F764–F767.

Yongue, B. G. & Roy, E. J. (1987). Endogenous aldosterone and corticosterone in brain cell nuclei of adrenal-intact rats: regional distribution and effects of physiological variations in serum steroids. *Brain Research* **436,** 49–61.

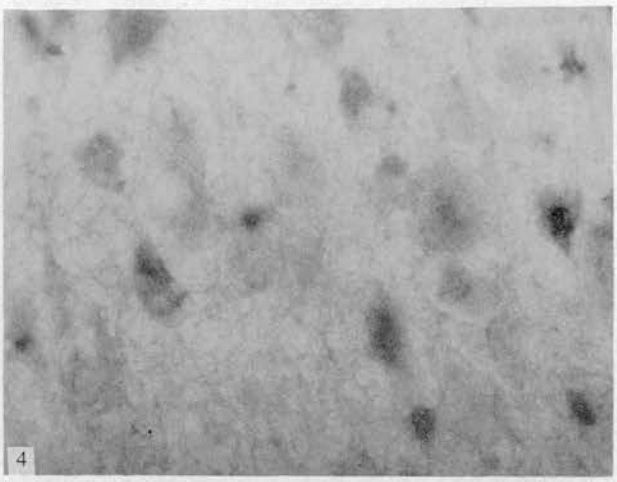
## DESCRIPTION OF PLATE

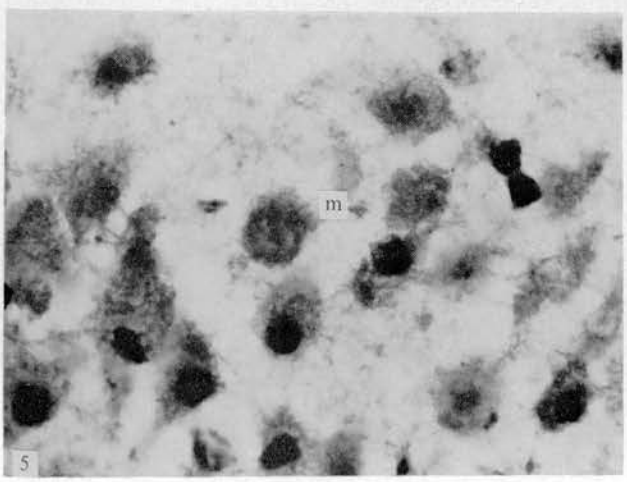
High magnification ( × 650) views of paraventricular nucleus showing in-situ hybridization with biotinylated cRNA probes. Expression of 11β-hydroxysteroid dehydrogenase (11β-OHSD) mRNA in (Fig. 1) magnocellular and (Fig. 2) parvicellular paraventricular nucleus; (Fig. 3) control section incubated with biotinylated 11β-OHSD 'sense' RNA probe and (Fig. 4) control section incubated with hybridization buffer without cRNA probe showing low 'endogenous biotin' signal. Mineralocorticoid receptor (MR) mRNA in expression in (Fig. 5) magnocellular and (Fig. 6) parvicellular paraventricular nucleus. Note presence of dark peroxidase product, derived from hybridization of biotinylated cRNA antisense probes to respective mRNAs, in both magnocellular (m) and parvicellular (p) neurones. Nuclei are counterstained with methyl green.

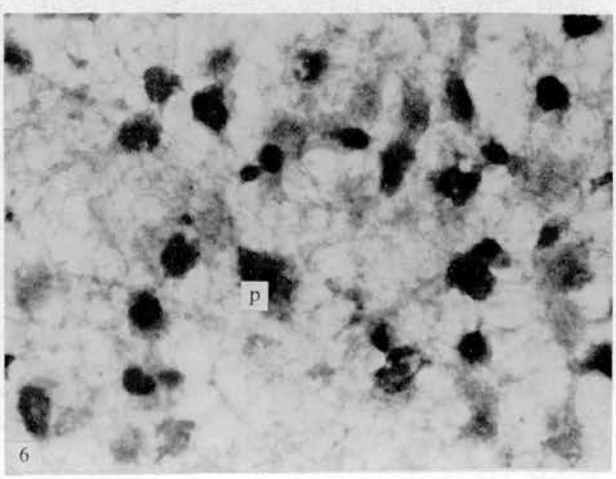







(Facing p. 478)

# Regulation of 11β-hydroxysteroid dehydrogenase by sex steroids *in vivo*: further evidence for the existence of a second dehydrogenase in rat kidney

**S. C. Low, S. N. Assaad, V. Rajan, K. E. Chapman, C. R. W. Edwards** and **J. R. Seckl**

Department of Medicine, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU, U.K.

REVISED MANUSCRIPT RECEIVED 19 April 1993

## ABSTRACT

11β-Hydroxysteroid dehydrogenase (11β-OHSD) catalyses the reversible conversion of corticosterone to inactive 11-dehydrocorticosterone, thus regulating glucocorticoid access to mineralocorticoid and perhaps glucocorticoid receptors *in vivo*. 11β-OHSD has been purified from rat liver and an encoding cDNA isolated from a liver library. However, several lines of indirect evidence suggest the existence of at least two isoforms of 11β-OHSD, one found predominantly in glucocorticoid receptor-rich tissues and the other restricted to aldosterone-selective mineralocorticoid target tissues and placenta. Here we have examined the effects of chronic (10 day) manipulations of sex-steroid levels on 11β-OHSD enzyme activity and mRNA expression in liver, kidney and hippocampus and present further evidence for the existence of a second 11β-OHSD isoform in kidney.

Gonadectomized male and female rats were given testosterone, oestradiol or blank silicone elastomer capsules, controls were sham-operated. In male liver, gonadectomy+oestradiol treatment led to a dramatic decrease in both 11β-OHSD activity (69 ± 8% decrease) and mRNA expression (97 ± 1% decrease). Gonadectomy and testosterone replacement had no effect on male liver 11β-OHSD. However, in female liver, where 11β-OHSD activity is approximately 50% of that in male liver, gonadectomy resulted in a marked increase in 11β-OHSD activity (120 ± 37% rise), which was reversed by oestradiol replacement but not testosterone treatment.

In male kidney, gonadectomy+oestradiol treatment resulted in a marked increase in 11β-OHSD activity (103 ± 4% rise). By contrast, 11β-OHSD mRNA expression was almost completely repressed (99 ± 0·1% decrease) by oestradiol treatment. This effect of oestradiol was reflected in a loss of 11β-OHSD mRNA in all regions of the kidney showing high expression by in-situ hybridization. In female kidney, oestradiol replacement also led to an increase in 11β-OHSD activity (70 ± 15% rise) while mRNA expression fell by 95 ± 3%. None of the treatments had any effect on enzyme activity or mRNA expression in the hippocampus, although transcription starts from the same promoter as liver.

We conclude that (i) sex steroids regulate 11β-OHSD enzyme activity and mRNA expression in a tissue-specific manner and (ii) the concurrence of increased enzyme activity with near absent 11β-OHSD mRNA expression in the kidney following oestradiol treatment suggests that an additional gene product is responsible, at least in part, for the high renal activity observed.

*Journal of Endocrinology* (1993) **139,** 27–35

## INTRODUCTION

11β-Hydroxysteroid dehydrogenase (11β-OHSD) is an intracellular enzyme, found in almost all tissues (Monder & Shackleton, 1985), which catalyses the reversible conversion of corticosterone to inactive 11-dehydrocorticosterone in the rat, or cortisol to cortisone in man (Lakshmi & Monder, 1985). 11β-OHSD activity is high in aldosterone-selective mineralocorticoid tissues such as kidney, where, despite a 100- to 1000-fold molar excess of circulating corticosterone, the otherwise non-selective mineralocorticoid receptor in the distal convoluted tubules and cortical collecting ducts selectively binds aldosterone due to the activity of 11β-OHSD (Edwards *et al.* 1988; Funder *et al.* 1988). 11β-OHSD activity is also present

  *Printed in Great Britain*
0022–0795/93/0139–0027 $08.00/0

in tissues containing predominantly glucocorticoid receptors, including, liver, colon (Fuller & Verity, 1990), skin (Teelucksingh *et al.* 1990) and cerebellum (Moisan *et al.* 1990*a*), and in tissues which express mineralocorticoid receptors but respond to physiological glucocorticoids *in vivo*, such as hippocampus (Reul & DeKloet, 1985; Moisan *et al.* 1990*b*). Recently, a cDNA encoding 11β-OHSD activity has been isolated from a rat liver library (Agarwal *et al.* 1989) and this has been used to demonstrate the presence of a single 11β-OHSD mRNA species in the majority of tissues including liver, but multiple species in kidney (Krozowski *et al.* 1990). Furthermore, we have demonstrated tissue-specific promoter usage of the rat 11β-OHSD gene leading to a single major transcript in liver, but two additional transcripts in the kidney, explaining the multiple RNA species detected (Moisan *et al.* 1992*a*). 11β-OHSD also exhibits tissue-specific ontogenesis (Moisan *et al.* 1992*b*), suggesting that 11β-OHSD is not constitutive but regulated. Several previous studies have examined sex-steroid regulation of 11β-OHSD activity in liver and kidney (Ghraf *et al.* 1975; Lax *et al.* 1978; Smith & Funder, 1991) but since 11β-OHSD activity could result from a number of enzyme isoforms, these studies are no longer definitive. The aim of this study was, therefore, to examine the effects of sex steroids on 11β-OHSD activity and mRNA encoding the liver 11β-OHSD isoform and to correlate mRNA levels (all hybridizing species) with enzyme activity in liver, kidney and hippocampus following chronic (10 day) sex-steroid treatments in male and female rats.

## MATERIALS AND METHODS

### In-vivo studies

Male and female rats (Han Wistar, 200–250 g) were maintained under conditions of controlled lighting (lights on from 07.00 to 19.00 h) and temperature (22 °C) with water and food available *ad libitum*. Groups of animals ($n=5$–6 per group) were gonadectomized or sham-operated under halothane anaesthesia. Gonadectomized animals were implanted subdermally with silicone elastomer capsules (1·98 mm internal diameter, 3·17 mm external diameter) containing testosterone propionate or 17β-oestradiol, which produce levels of testosterone (Naville *et al.* 1992) and oestradiol (Painson *et al.* 1991) not significantly different from those found in the plasma of control males and females. The remainder and sham-operated animals received blank capsules. Rats were killed 10 days after surgery.

### Assay of 11β-OHSD enzyme activity

Rats were decapitated and the hippocampus, liver and kidneys removed and dissected on ice. Tissues were homogenized in Krebs–Ringer bicarbonate buffer (118 mmol NaCl/l, 3·8 mmol KCl/l, 1·19 mmol $KH_2PO_4$/l, 2·54 mmol $CaCl_2 . 2H_2O$/l, 1·19 mmol $MgSO_4 . 7H_{23}O$/l, 25 mmol $NaHCO_3$/l and 0·2% glucose, pH 7·4) and assayed as described previously (Moisan *et al.* 1990*a*). Briefly, after estimating the total protein concentration colorimetrically (Bio-Rad protein assay kit, Hemel Hempstead, Herts, U.K.), an aliquot of homogenate was incubated with 200 μmol $NADP^+$/l and 12 nmol [1,2,6,7 $^3$H]corticosterone/l (specific activity, 88 Ci/mmol; Amersham International plc, Amersham, Bucks, U.K.) in Krebs–Ringer buffer (+0·2% bovine serum albumin) for 10 min at 37 °C. The protein concentration in each reaction was adjusted such that additional protein produced a linear increase in 11β-OHSD enzyme activity. This was found to be 50 mg/l liver, 25 mg/l kidney and 500 mg/l hippocampus. After incubation, steroids were extracted with ethyl acetate and separated by thin-layer chromatography. The conversion of [$^3$H]corticosterone to [$^3$H]11-dehydrocorticosterone was calculated from the radioactivity of each fraction.

### Analysis of mRNA

Total RNA was extracted from hippocampus, liver and kidney by the guanidinium thiocyanate method as described (Chomczynski & Sacchi, 1987). Briefly, tissue was homogenized in 4 mol guanidinium thiocyanate/l, 0·025 mol sodium citrate/l, 0·5% sarcosyl and 0·1 mol β-mercaptoethanol/l. DNA was precipitated by the addition of 0·2 mol sodium acetate/l (pH 4) and protein removed by phenol–chloroform extraction. The aqueous phase containing RNA was precipitated twice with isopropanol and resuspended in diethylpyrocarbonate-treated water. RNA concentration and purity was assayed spectrophotometrically and aliquots stored at −70 °C prior to use. Aliquots of total RNA from hippocampus (15 μg), liver (10 μg) and kidney (10 μg) were fractionated on 1·2% agarose–2·2 mol formaldehyde/l gels and blotted onto nitrocellulose or nylon (Hybond C extra or Hybond N, Amersham International plc) by capillary transfer at 4 °C overnight. Membranes were prehybridized in 50% formamide, 5 × SSPE (20 × SSPE = 6 mol NaCl/l, 0·18 mol $NaH_2PO_4$/l, 20 mmol EDTA/l; pH 7·4), 5 × Denhardt's solution (5 × Denhardt's = 0·1% bovine serum albumin, 0·1% Ficoll, 0·1% polyvinylpyrolidone), 0·2 g denatured herring testes DNA/l, 0·1% sodium dodecyl sulphate (SDS) and 1 mmol EDTA/l. Hybridization was

performed in identical buffer containing radiolabelled 11β-OHSD probe. A 1·2 kb EcoR1 fragment encoding 11β-OHSD cDNA (Agarwal *et al.* 1989) was labelled with [$^{32}$P]dCTP (3000 Ci/mmol; Amersham International plc) to a specific activity of 1–2 × $10^9$ c.p.m./μg DNA and diluted in hybridization buffer (3–6 × $10^6$ c.p.m./ml). Hybridization was performed at 42 °C overnight and the membrane was washed to a final stringency of 0·2 × SSC (1 × SSC = 0·5 mol NaCl/l and 0·015 mol sodium citrate/l), 0·1% SDS at 60 °C and exposed to Kodak XAR film for varying lengths of time adjusted to the linear range of the film. Filters were stripped and rehybridized under identical conditions with similarly labelled 7S cDNA probes (Balmain *et al.* 1982) which does not vary in non-dividing cells (Seckl & Fink, 1992). Films were quantified by computer densitometry (Seescan, Cambridge, Cambs, U.K.).

### In-situ hybridization

Male rats were gonadectomized and implanted with silicone elastomer capsules containing oestradiol or blank. Ten days later they were decapitated and the brains rapidly removed and frozen on dry ice. Coronal sections (10 μm) were mounted onto gelatin-coated poly-L-lysine-treated slides and stored at − 70 °C prior to hybridization as previously described (Moisan *et al.* 1990*b*). Briefly, tissue sections were post-fixed in 4% paraformaldehyde/0·1 mol phosphate buffer/l and washed in 2 × SSC. T3 RNA polymerase (NBL, Cramlington, Northumberland, U.K.) was used to transcribe 597 bp $^{35}$S-labelled antisense cRNA probes from StyI linearized Bluescript vectors containing the 11β-OHSD cDNA insert (Agarwal *et al.* 1989). The [$^{35}$S]cRNA probe was denatured and added at a final concentration of 10 × $10^6$ c.p.m./ml to hybridization buffer (50% formamide, 0·6 mol NaCl/l, 10 mmol Tris–HCl/l (pH 7·5), 0·02% Ficoll, 0·02% polyvinylpyrrolidine, 0·1% bovine serum albumin, 1 mmol EDTA/l, 0·1 g denatured salmon sperm DNA/l, 0·5 g total yeast tRNA/l, 0·1 mg dextran sulphate/l and 10 mmol dithiothreitol/l) and applied to the sections. Slides were incubated overnight in sealed boxes at 50 °C. Following hybridization, slides were rinsed twice in 2 × SSC for 15 min at room temperature prior to RNase A digestion (30 mg/l for 45 min at 37 °C). Slides were washed to a maximum stringency of 0·1 × SSC at 60 °C for 1 h. After air drying, sections were exposed to Hyperfilm βmax (Amersham International plc) for 10 days and developed. To demonstrate specificity of the 11β-OHSD probe, sections were hybridized with a sense probe (10 × $10^6$ c.p.m./ml) transcribed with T7 RNA polymerase (NBL).

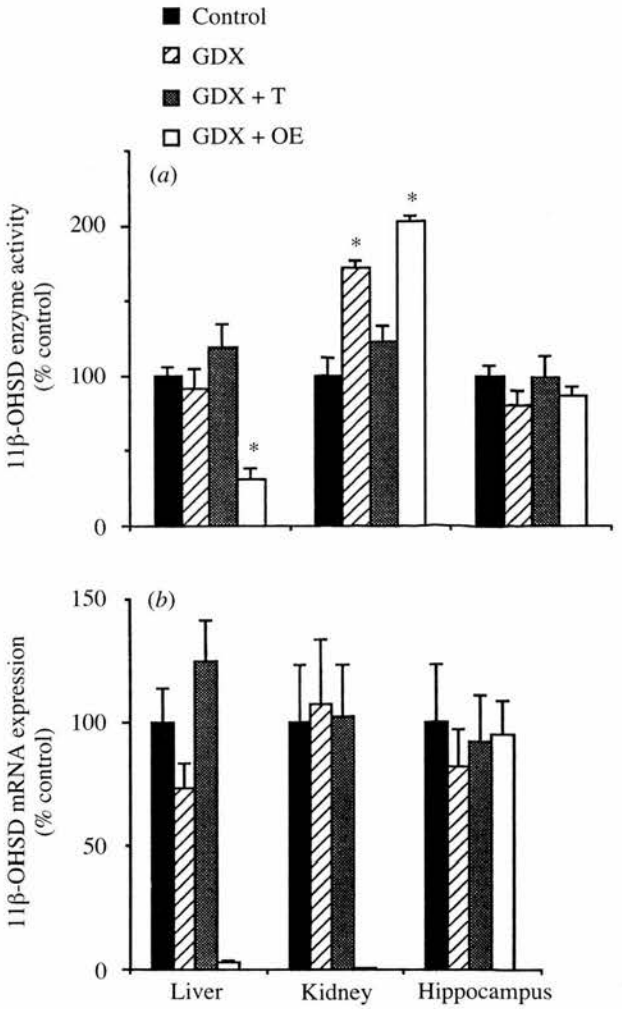



FIGURE 1. Effect of chronic (10 days) sex-steroid manipulations on (*a*) 11β-hydroxysteroid dehydrogenase (11β-OHSD) enzyme activity and (*b*) 11β-OHSD mRNA expression in male rat liver, kidney and hippocampus. Data are expressed as a percentage of activity or mRNA levels in sham-operated controls. GDX = gonadectomy, GDX + T = gonadectomy + testosterone replacement, GDX + OE = gonadectomy + oestradiol treatment. *$P$<0·05 compared with sham-operated controls (Student's $t$-test).

### Primer extension and RNase protection analyses

A synthetic oligonucleotide complimentary to bases +74 to +36 (Agarwal *et al.* 1989; Moisan *et al.* 1992*a*) was 5′ end-labelled with $^{32}$P-γ-[ATP] (Amersham International plc) and T4 polynucleotide kinase (Boehringer Mannheim, Lewes, E. Sussex, U.K.) to a high specific activity (1–2 × $10^9$ c.p.m./mg). Approximately 5 × $10^5$ c.p.m. were hybridized in solution to total hippocampal (100 μg), liver (20 μg) and kidney (20 μg) RNA samples at 60 °C for 1 h in annealing buffer (100 mmol KCl/l, 10 mmol $MgCl_2$/l, 25 mmol

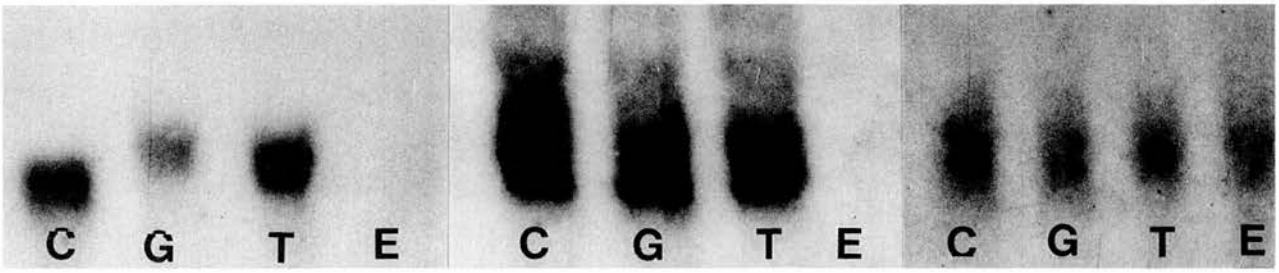



FIGURE 2. Northern blot autoradiograph hybridized with $^{32}$P-labelled 11β-hydroxysteroid dehydrogenase (11β-OHSD) cDNA probe showing the effects of sham-operation (C), gonadectomy (G), gonadectomy+testosterone replacement (T), gonadectomy+oestradiol treatment (E) for 10 days on 11β-OHSD mRNA expression in male rat liver, kidney and hippocampus.

Tris–HCl/l, pH 8·5) in a final volume of 12 μl. Primer extension reactions were carried out in 40 μl of 30 mmol KCl/l, 8 mmol $MgCl_2$/l, 50 mmol Tris–HCl/l, 500 μmol each dNTP/l, 50 g actinomycin D/l, 20 units RNase inhibitor (NBL) and 50 units of AMV reverse transcriptase (Promega, Southampton, Hants, U.K.) at 42 °C for 60 min. After phenol–chloroform extraction and ethanol precipitation, the size of the reaction product was estimated on a 6% denaturing polyacrylamide gel by comparison with a sequencing reaction.

For RNase protection, $^{32}$P[CTP]-labelled antisense RNA probes were synthesized from linear DNA templates using either T7 or T3 RNA polymerases. Templates were degraded using DNase I. RNA probe ($1{\cdot}5 \times 10^6$ c.p.m.) was hybridized to hippocampal (70 μg), liver (20 μg) and kidney (20 μg) RNA samples overnight at 47 °C in 80% formamide, 0·4 mol NaCl/l, 40 mmol Pipes/l (pH 6·7) and 1 mmol EDTA/l. Non-hybridized RNA was degraded by the addition of RNase A (40 mg/l) and T1 (2 mg/l) for 1 h at 30 °C. Following proteinase K treatment and phenol–chloroform extraction, the samples were ethanol-precipitated and analysed on a 6% polyacrylamide gel in parallel with a sequencing reaction.

### Statistics

Data were compared by analysis of variance (ANOVA) followed by Student's unpaired *t*-test. Significance was set at $P<0{\cdot}05$. Values are expressed as means ± S.E.M.

## RESULTS

### Liver

In male rats, gonadectomy alone and following testosterone replacement did not affect hepatic 11β-OHSD activity (Fig. 1*a*). However, oestradiol administration for 10 days produced a large decrease in enzyme activity of 69 ± 8% in male liver (Fig. 1*a*). In parallel, oestradiol administration for 10 days led to a dramatic decrease in 11β-OHSD mRNA expression of 97 ± 1%. As with enzyme activity, no other manipulation altered 11β-OHSD mRNA expression (Figs 1*b* and 2).

In female rats in contrast (where 11β-OHSD activity is approximately 50% of that expressed in male liver), gonadectomy resulted in a marked increase in 11β-OHSD activity (120 ± 37% increase) which was not affected by testosterone treatment (108 ± 24% increase compared with controls) but was reversed by oestradiol replacement (Fig. 3*a*). However, ovariectomy either alone or following testosterone treatment had no effect on 11β-OHSD mRNA expression, while oestradiol replacement resulted in a significant decrease in mRNA levels (to 82 ± 10% of control levels) (Fig. 3*b*).

### Kidney

Sex-steroid manipulations were associated with marked changes in both 11β-OHSD enzyme activity and mRNA expression in kidney. Gonadectomy of male rats resulted in a rise in enzyme activity of 72 ± 5% (Fig. 1*a*). Testosterone replacement prevented the gonadectomy-induced increase in enzyme activity by 10 days (Fig. 1*a*). Oestradiol treatment of gonadectomized rats led to a large increase in 11β-OHSD activity (103 ± 4% rise) (Fig. 1*a*). In striking contrast, 11β-OHSD mRNA expression was virtually undetectable after 10 days (Figs 1*b* and 2). The oestradiol-induced suppression of 11β-OHSD gene transcripts affected all mRNA species expressed in the kidney.

In female kidney, 11β-OHSD activity was increased by 10 days of oestradiol replacement (91 ± 15% rise)

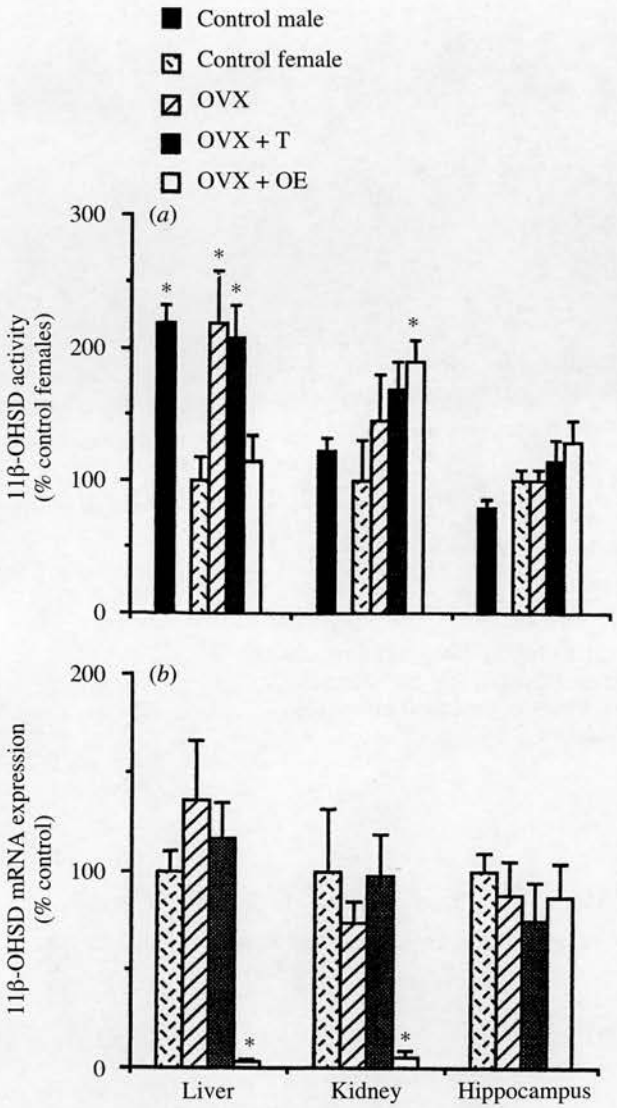



FIGURE 3. Effect of chronic (10 day) sex-steroid manipulations on (*a*) 11β-hydroxysteroid dehydrogenase (11β-OHSD) enzyme activity and (*b*) 11β-OHSD mRNA expression in female rat liver, kidney and hippocampus. Data are expressed as a percentage of activity or mRNA levels in sham-operated controls. OVX=ovariectomy, OVX+T=ovariectomy with testosterone treatment, OVX+OE=ovariectomy with oestradiol replacement. *$P<0{\cdot}05$ compared with sham-operated controls (Student's *t*-test).

but not by ovariectomy either alone or following testosterone treatment, in parallel to the changes in 11β-OHSD activity in males (Fig. 3*a*). Again, oestradiol treatment resulted in a dramatic decrease in 11β-OHSD mRNA levels (89 ± 6% decrease) despite the induction of 11β-OHSD activity (Fig. 3*b*).

### Hippocampus

None of the sex-steroid manipulations had any effect on hippocampal 11β-OHSD activity (Figs 1*a* and 3*a*) or mRNA expression (Figs 1*b*, 2 and 3*b*) in male or female rats.

### In-situ hybridization of 11β-OHSD mRNA in kidney

Ten days after gonadectomy 11β-OHSD mRNA was highly expressed in the kidney, localized predominantly to the inner cortex and outer medulla, as previously shown in control kidney (Yau *et al.* 1991). Oestradiol treatment for 10 days markedly attenuated 11β-OHSD mRNA expression in all kidney regions (Fig. 4). Controls hybridized with sense RNA showed no specific hybridization (data not shown).

### Identification of 11β-OHSD transcription start sites

To eliminate the possibility that the lack of regulation by sex steroids in hippocampus was due to differential promoter regulation, primer extension analysis was carried out on RNA isolated from the hippocampus from untreated animals to determine the start point(s) of transcription. Extension of the oligonucleotide (complimentary to bases +74 to +36 within exon 1) indicated that the major start site in the hippocampus is the same as the major start site in liver – and is also used in the kidney (data not shown). These findings were confirmed using RNase protection (Fig. 5). The hippocampal transcription start site is 105 base pairs 5′ of the start of translation in the liver in agreement with published findings (Agarwal *et al.* 1989). This result, taken with the presence of a single band on Northern analysis, indicates that the +1 transcription start site in liver is also used in the hippocampus.

## DISCUSSION

11β-OHSD has previously been shown to be regulated in a complex manner by various hormones including thyroxine (Hellman *et al.* 1961; Koerner & Hellman, 1964; Lax *et al.* 1979; Zumoff *et al.* 1983), insulin (Hammami & Siiteri, 1991; Siiteri *et al.* 1991) and glucocorticoids (Moisan *et al.* 1990*c*; Hammami *et al.* 1991; Smith & Funder 1991). Here we demonstrate that hepatic 11β-OHSD enzyme activity and mRNA expression are sexually dimorphic, showing repression by oestradiol treatment, whereas gonadectomy or testosterone replacement have no effect on either enzyme activity or mRNA levels in males, while gonadectomy in females results in an induction of 11β-OHSD activity which is reversed by oestradiol replacement. Hippocampal 11β-OHSD enzyme

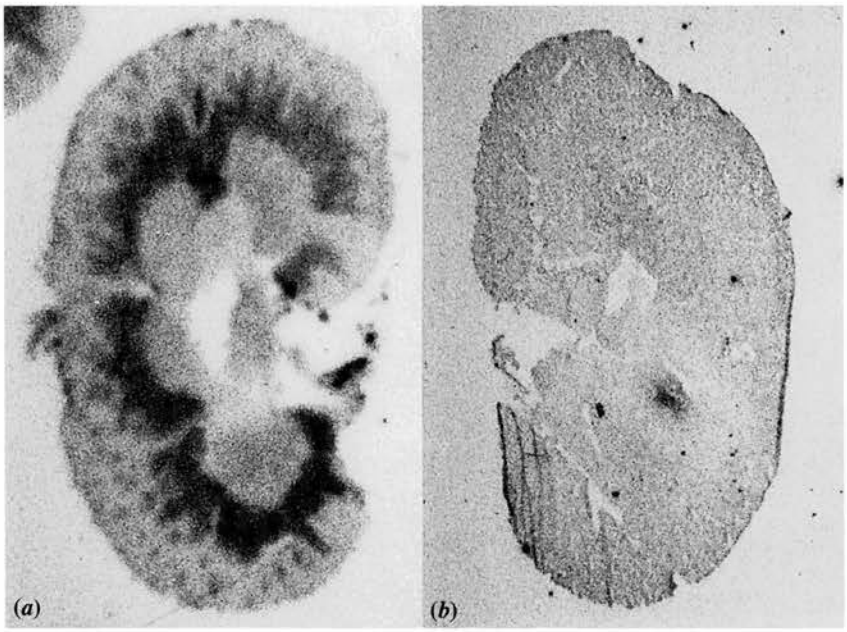



FIGURE 4. Autoradiograph of longitudinal rat kidney sections hybridized with $^{35}$S-labelled 11β-hydroxysteroid dehydrogenase (11β-OHSD) cRNA probes showing 11β-OHSD mRNA expression following (*a*) gonadectomy or (*b*) gonadectomy+oestradiol treatment for 10 days.

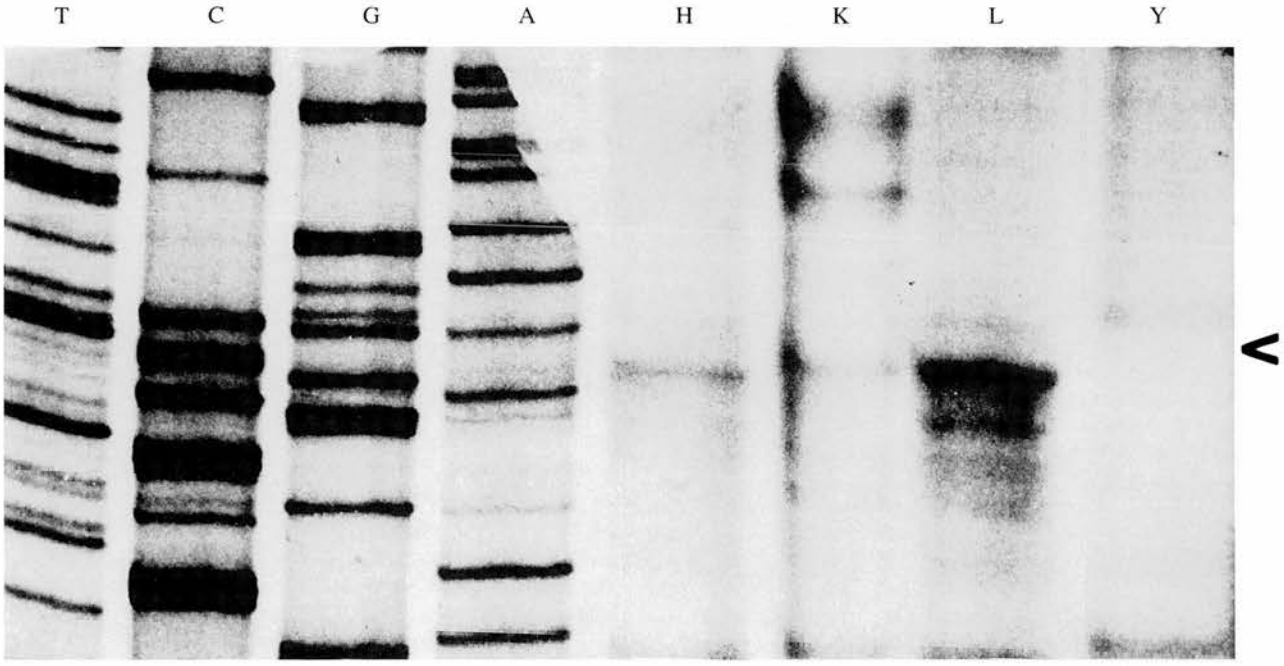



FIGURE 5. RNase protection assay. L=liver total RNA; K=kidney total RNA; H=hippocampal total RNA; Y=yeast RNA; run with a sequencing reaction. The arrowhead indicates the presence of the major transcription start site (+1) at the same position in liver and hippocampus. This start site is also used in kidney.

activity and mRNA expression are not affected by any of the sex-steroid manipulations employed. In contrast to liver and hippocampus, changes in renal 11β-OHSD mRNA expression do not parallel enzyme activity; oestradiol treatment results in near complete loss of 11β-OHSD mRNA expression but induction of enzyme activity in both males and females.

Previous studies have shown 11β-OHSD enzyme activity to be regulated by sex steroids in liver (Lax *et al.* 1978) and kidney (Smith & Funder, 1991) and that 11β-OHSD enzyme activity is higher in male than female rat liver (Hoff *et al.* 1973; Lax *et al.* 1979). These studies could not address changes in 11β-OHSD mRNA expression. We found that following chronic (10 days) administration of oestradiol to gonadectomized male rats, liver 11β-OHSD enzyme activity was significantly decreased, in agreement with previous work (Lax *et al.* 1978). We have also shown that oestradiol replacement in female rat liver reverses the gonadectomy-induced increase in 11β-OHSD activity. In addition, oestradiol treatment markedly decreased liver 11β-OHSD mRNA expression in both males and females, suggesting that oestrogen represses 11β-OHSD biosynthesis *in vivo*, explaining the higher basal levels of 11β-OHSD activity in male liver (Lax *et al.* 1978).

In contrast to liver, hippocampal 11β-OHSD enzyme activity and mRNA expression were not altered by any of the sex-steroid manipulations employed. This difference is unlikely to be due to failure of access of oestradiol or testosterone to the brain since both steroids readily cross the blood–brain barrier and bind to central receptors when administered peripherally. Furthermore, although the documented tissue-specific promoter usage of the 11β-OHSD gene (Moisan *et al.* 1992*a*) might explain the absence of sex-steroid regulation in hippocampus, we found that liver and hippocampus predominantly employ the same promoter, making this unlikely. However, sexually dimorphic expression of many genes and gene products is thought to be due to an indirect effect of sex steroids acting on the secretory profiles of growth hormone, which differ in male and female rats (Saunders *et al.* 1976; Tannenbaum & Martin, 1976). Thus, genes encoding the major urinary proteins of mice are expressed at five- to ten-times higher levels in male than female mouse liver (Hastie *et al.* 1979; McIntosh & Bishop, 1989); this dimorphism can be reversed by administration of testosterone to females (Szoka & Paigen, 1978; Clissold *et al.* 1984) and represents an indirect effect of testosterone acting to modify growth hormone secretory patterns (Knopf *et al.* 1983; Norstedt & Palmiter, 1984). Similarly, testosterone induction of the hepatic isoform of 3β-hydroxysteroid dehydrogenase is mediated by androgen suppression of growth hormone and can be reversed by high constant (female-type) growth hormone replacement (Naville *et al.* 1991). Since the effects of oestradiol on 11β-OHSD activity in kidney require an intact hypophysis (Ghraf *et al.* 1975; Lax *et al.* 1978), the actions of sex steroids on 11β-OHSD may also be growth hormone-mediated. The lack of regulation of hippocampal 11β-OHSD by sex steroids may reflect the failure of peripheral growth hormone, as with many other peptides, to access the brain.

Recent work has shown that kidney 11β-OHSD enzyme activity in increased by chronic (10 day) oestradiol treatment, but is not regulated by gonadectomy or testosterone replacement (Smith & Funder, 1991). We now show that gonadectomy induces 11β-OHSD activity in males, an effect which is reversed by testosterone replacement, and that chronic oestradiol administration markedly induces 11β-OHSD enzyme activity, but almost completely represses 11β-OHSD mRNA expression in both males and females. There are several possible explanations for the discrepancy between renal 11β-OHSD mRNA expression and enzyme activity. (i) This might represent differences in turnover/half-life of mRNA and protein. However, given the degree of transcriptional repression it is very unlikely that increased enzyme activity represents enhanced translation of any remaining mRNA. Nor, at least from the liver 11β-OHSD data presented here, do such gross differences between mRNA and protein half-life seem likely explanations for the opposite changes in mRNA levels and enzyme activity seen in kidney. (ii) Previous studies using 11β-OHSD cDNA probes encoding 'liver-type' 11β-OHSD have shown multiple mRNA species in the kidney (Krozowski *et al.* 1990), including a cDNA encoding a truncated protein which contains the putative active sites of the enzyme. However, it appears unlikely that any one of these mRNA species is responsible for the high enzyme activity in the kidney since all result from differential promoter usage of the same gene (Moisan *et al.* 1992*a*) and all were repressed following oestradiol treatment. (iii) Alternatively, a second gene product might be responsible for oestradiol-induced renal 11β-OHSD enzyme activity. The second gene would presumably produce mRNA which hybridizes very weakly or not at all on Northern analysis with the cloned 'liver-type' 11β-OHSD cDNA. This hypothesis is supported by several studies which suggest the presence of a second 11β-OHSD in kidney. Western blots have demonstrated three immunoreactive components in the kidney (Monder & Lakshmi, 1990), while kinetic studies have indicated the presence of an 11β-OHSD with a $K_m$ two orders of magnitude lower than the hepatic species (Naray-Fejes-Toth *et al.* 1991). Histochemical studies performed on rat kidney have identified an $NAD^+$-requiring species (Mercer & Krozowski, 1991), while we have shown $NAD^+$-

dependence of human and rat placental and rat renal 11β-OHSD and have, in addition, partially purified the human placental isoform of 11β-OHSD, which has a much higher glucocorticoid affinity ($K_m$ low nmol/l) than the liver isoform, suggesting that the $NAD^+$-dependent enzyme present in rat kidney may be more suited to 'protect' renal mineralocorticoid receptors from circulating glucocorticoids (Brown *et al.* 1993*a,b*). Finally, during development, 11β-OHSD activity in the kidney is high from postnatal day 1 whereas 'liver-type' 11β-OHSD mRNA expression is low and does not reach adult levels until postnatal day 15 (Moisan *et al.* 1992*b*).

These experiments demonstrate that 11β-OHSD is regulated by sex steroids in a tissue-specific manner. The discrepancy between oestradiol-mediated induction of 11β-OHSD activity in the kidney in the face of near complete repression of all known mRNA transcripts of the cloned 'liver-type' 11β-OHSD gene lend strong support to suggestions that more than one gene encodes 11β-OHSD activities. The nature of the oestrogen-induced renal enzyme remains to be determined.

## ACKNOWLEDGEMENTS

We thank Drs C. Monder and P. White for generously providing the full-length rat 11β-OHSD cDNA clone and J. Noble for technical assistance. This work was supported by a Wellcome Trust/Royal Society of Edinburgh Senior Research Fellowship (J.R.S.), a Caledonian Research Foundation/Royal Society of Edinburgh Fellowship (K.E.C.) and grants from the Wellcome Trust, Scottish Hospital Endowments Research Trust and the Sir Stanley and Lady Davidson Research Fund (to J.R.S. and C.R.W.E.).

## REFERENCES

Agarwal, A. K., Monder, C., Eckstein, B. & White, P. C. (1989). Cloning and expression of rat cDNA encoding corticosteroid 11β-dehydrogenase. *Journal of Biological Chemistry* **264,** 18939–18943.

Balmain, A., Krumlauf, R., Vass, J. K. & Birnie, G. D. (1982). Cloning and characterisation of the abundant cytoplasmic 7S RNA from mouse cells. *Nucleic Acids Research* **10,** 4259–4277.

Brown, R. W., Chapman, K. E., Edwards, C. R. W. & Seckl, J. R. (1993*a*). Human placental 11β-hydroxysteroid dehydrogenase: Evidence for and partial purification of a distinct NAD-dependent isoform. *Endocrinology* **132,** 2614–2621.

Brown, R. W., Chapman, K. E., Edwards, C. R. W. & Seckl, J. R. (1993*b*). A novel high affinity 11β-hydroxysteroid dehydrogenase in placenta and kidney? *Journal of Endocrinology* **137** (Suppl.), Abstract No. P18.

Chomczynski, P. & Sacchi, N. (1987). Single step method of RNA isolation by guanidinium thiocyanate-phenol-choloroform extraction. *Analytical Biochemistry* **162,** 156–159.

Clissold, P. M., Hainey, S. & Bishop, J. O. (1984). Messenger RNAs coding for mouse major urinary proteins are differentially induced by testosterone. *Biochemical Genetics* **22,** 379–387.

Edwards, C. R. W., Stewart, P. M., Burt, D., Brett, L., McIntyre, M. A., Sutanto, W. S., DeKloet, E. R. & Monder, C. (1988). Localisation of 11β-hydroxysteroid dehydrogenase – tissue specific protector of the mineralocorticoid receptor. *Lancet* **ii,** 986–989.

Fuller, P. J. & Verity, K. (1990). Colonic sodium-potassium adenosine triphosphate subunit gene expression: ontogeny and regulation by adrenocortical steroids. *Endocrinology* **127,** 32–38.

Funder, J. W., Pearce, P. T., Smith, R. & Smith, A. I. (1988). Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science* **242,** 583–585.

Ghraf, R., Lax, E. R., Hoff, H.-G. & Schreifers, H. (1975). The role of the gonads and the hypophysis in the regulation of hydroxysteroid dehydrogenase activities in the rat. *Hoppe–Seylers Zeitschrift für Physiologische Chemie* **356,** 135–142.

Hammami, M. M. & Siiteri, P. K. (1991). Regulation of 11β-hydroxysteroid dehydrogenase activity in human skin fibroblasts: enzymatic modulation of glucocorticoid action. *Journal of Clinical Endocrinology and Metabolism* **73,** 326–334.

Hastie, N. D., Held, W. A. & Toole, J. J. (1979). Multiple genes coding for the androgen regulated major-urinary proteins of the mouse. *Cell* **17,** 449–457.

Hellman, L., Bradlow, H. L., Zumoff, B. & Gallagher, T. F. (1961). The influence of thyroid hormone administration on the 11β-hydroxysteroid dehydrogenase mRNA expression in rat kidney. *Journal of Clinical Endocrinology and Metabolism* **21,** 1231–1247.

Hoff, H. G., Ghraff, R., Raible, M. & Schreifers, H. (1973). Ontogenesis of hydroxysteroid dehydrogenase activities in rat liver. Age dependent and sexual differentiation in the cytosol and microsomal fraction. *Hoppe–Seylers Zeitschrift für Physiologische Chemie* **354,** 306–331.

Knopf, J. L., Gallagher, J. F. & Held, W. A. (1983). Differential multi-hormone regulation of the mouse major urinary protein gene family in the liver. *Molecular and Cellular Biology* **3,** 2232–2240.

Koerner, D. R. & Hellman, L. (1964). Effect of thyroxine administration on the 11β-hydroxysteroid dehydrogenases in rat liver and kidney. *Endocrinology* **75,** 592–601.

Krozowski, Z. S., Stuchbery, S., White, P. C., Monder, C. & Funder, J. W. (1990). Characterisation of 11β-hydroxysteroid dehydrogenase gene expression: identification of multiple unique forms of messenger ribonucleic acid in the rat kidney. *Endocrinology* **129,** 17–21.

Lakshmi, V. & Monder, C. (1985). Evidence for independent 11-oxidase and 11-reductase activities of 11β-hydroxysteroid dehydrogenase: enzyme latency, phase transition and lipid requirements. *Endocrinology* **116,** 552–560.

Lax, E. R., Ghraf, R. & Schreifers, H. (1978). The hormonal regulation of hepatic microsomal 11β-hydroxysteroid dehydrogenase activity in the rat. *Acta Endocrinologica* **89,** 352–358.

Lax, E. R., Ghraf, R., Schreifers, H. & Voigt, K.-H. (1979). The involvement of the thyroid and adrenal in the regulation of enzyme activities of hepatic and renal steroid metabolism in the rat. *Hoppe–Seylers Zeitschrift für Physiologische Chemie* **360,** 137–143.

McIntosh, I. & Bishop, O. (1989). Differential expression in male and female mouse liver of very similar mRNAs specified by two group 1 major-urinary protein genes. *Molecular and Cellular Biology* **9,** 2202–2207.

Mercer, W. & Krozowski, Z. S. (1991). Localisation of an 11β-hydroxysteroid dehydrogenase activity to the distal nephron. Evidence for the existence of two species of dehydrogenase in the rat kidney. *Endocrinology* **130,** 540–543.

Moisan, M.-P., Edwards, C. R. W. & Seckl, J. R. (1992*a*). Differential promoter usage by the rat 11β-hydroxysteroid dehydrogenase gene. *Molecular Endocrinology* **6,** 1082–1087.

Moisan, M.-P., Edwards, C. R. W. & Seckl, J. R. (1992*b*). Ontogeny of 11β-hydroxysteroid dehydrogenase in rat brain and kidney. *Endocrinology* **130,** 400–404.

Moisan, M.-P., Seckl, J. R., Brett, L. P., Monder, C., Agarwal, A. K., White, P. C. & Edwards, C. R. W. (1990*a*). 11Beta-hydroxysteroid dehydrogenase messenger ribonucleic acid expression, bioactivity and immunoreactivity in rat cerebellum. *Journal of Neuroendocrinology* **2,** 853–858.

Moisan, M.-P., Seckl, J. R. & Edwards, C. R. W. (1990*b*). 11β-Hydroxysteroid dehydrogenase bioactivity and messenger RNA expression in rat forebrain: localization in hypothalamus, hippocampus and cortex. *Endocrinology* **127,** 1450–1455.

Moisan, M.-P., Seckl, J. R. & Edwards, C. R. W. (1990*c*). Effect of adrenalectomy and dexamethasone administration on 11β-hydroxysteroid dehydrogenase *in vitro* activity in rat brain and kidney. *Journal of Physiology* **434,** 87P.

Monder, C. & Lakshmi, V. (1990). Corticosteroid 11β-dehydrogenase of rat tissues: immunological studies. *Endocrinology* **126,** 2435–2443.

Monder, C. & Shackleton, C. H. L. (1985). 11β-Hydroxysteroid dehydrogenase: fact or fancy? *Steroids* **44,** 383–415.

Naray-Fejes-Toth, A., Watlington, C. & Fejes-Toth, G. (1991). 11β-Hydroxysteroid dehydrogenase activity in the renal target cells of aldosterone. *Endocrinology* **129,** 17–21.

Naville, D., Keeney, D. S., Jenkin, G., Murry, B. A., Head, J. R. & Mason, J. I. (1991). Regulation of expression of male-specific rat liver microsomal 3β-hydroxysteroid dehydrogenase. *Molecular Endocrinology* **5,** 1090–1100.

Norstedt, G. & Palmiter, R. (1984). Secretory rhythm of growth hormone regulates sexual differentiation of mouse liver. *Cell* **36,** 805–812.

Painson, J.-C., Thorner, M. O., Kreig, R. J. & Tannenbaum, G. S. (1992). Short term adult exposure to estradiol feminises the male pattern of spontaneous and growth hormone-releasing factor-stimulated growth hormone secretion in the rat. *Endocrinology* **130,** 511–519.

Reul, J. M. H. M. & DeKloet, E. R. (1985). Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. *Endocrinology* **117,** 2505–2512.

Saunders, A., Terry, L. C., Audet, J., Brazeau, P. & Martin, J. B. (1976). Dynamic studies of growth hormone and prolactin secretion in the female rat. *Neuroendocrinology* **21,** 193–203.

Seckl, J. R. & Fink, G. (1992). Antidepressants increase glucocorticoid and mineralocorticoid receptor mRNA-expression in the rat hippocampus *in vivo*. *Neuroendocrinology* **55,** 621–626.

Siiteri, P. K., Hammami, M. M. & Murai, J. T. (1991). The free hormone hypothesis and in steroid resistant states. *Journal of Steroid Biochemistry and Molecular Biology* **36** (Suppl.), 107.

Smith, R. E. & Funder, J. W. (1991). Renal 11β-hydroxysteroid dehydrogenase activity: effects of age, sex and altered hormonal status. *Journal of Steroid Biochemistry and Molecular Biology* **38,** 265–267,

Szoka, P. & Paigen, K. (1978). Regulation of mouse major urinary protein production by the MUP-a gene. *Genetics* **90,** 597–612.

Tannenbaum, G. S. & Martin, J. B. (1976). Evidence for endogenous ultradian rhythm governing growth hormone secretion in the rat. *Endocrinology* **98,** 562–570.

Teelucksingh, S., Mackie, A., Burt, D., McIntyre, M., Brett, L. & Edwards, C. R. W. (1990). Potentiation of hydrocortisone activity in the skin by glycerrhetinic acid. *Lancet* **335,** 1060–1063.

Yau, J. L. W., van Haarst, A. D., Moisan, M.-P., Fleming, S., Edwards, C. R. W. & Seckl, J. R. (1991). 11β-Hydroxysteroid dehydrogenase mRNA expression in rat kidney. *American Journal of Physiology* **260,** F764–F767.

Zumoff, B., Bradlow, H. L., Levin, J. & Fukushima, D. K. (1983). Influence of thyroid function on the *in vivo* cortisol/cortisone equilibrium in man. *Journal of Steroid Biochemistry* **18,** 437–440.